

[illegible]

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
FAUOAGFFAZI	08}~æ	FAUOAGFFAZI AT OYAOFGA

Dose Estrogen-Induced Apoptosis	5b. GRANT NUMBER W81XWH-06-1-0590A
---------------------------------	--

6. AUTHOR(S)	5d. PROJECT NUMBER
Xiti k'Etck "lat'cen "ODG "Ri E "ELe "HO of Lek"	

Á 1	
	5f. WORK UNIT NUMBER

Georgetown University	NUMBER
-----------------------	--------

Q. I don't see any of the other things that are in the report, is that right?

12. DISTRIBUTION / AVAILABILITY STATEMENT	
---	--

$\frac{1}{n} \sum_{i=1}^n \log \left(\frac{\lambda_i}{\mu_i} \right) = \frac{1}{n} \sum_{i=1}^n \log \left(\frac{\lambda_i}{\mu_i} \right)$

15. SUBJECT TERMS
Gene expression microarrays, proteomics, c-Src inhibitor PP2, estrogen receptor complex

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON WUOT UT OAM
REPORT	ABSTRACT	THIS PAGE			19b. TELEPHONE NUMBER (include area code)

11

Vcdrg'qh'Eqpvgpvu'

Kpvt qf wevkapi í 5"

[illegible]

Vcum'3"

""""Vcum'3c"*NEEE"/'Kuccet+''

[illegible]

Vcum'4'''

""""Vcum'4c'*HEEE"/'LqtfcplCtkc/k+

Y qtm!Ceeqo r rkuj gf í í 0 í 007"

11

""Vcum'4d/3"*I W"/LqtfcpIHcp+''

[illegible][illegible]

!!

""Vcum'4d/4"*I W/"LqtfcpIHcp+"'

Kvtfwvqpi í 0034"

[illegible]

!!

""""Vcum'4d/5"*I W/"LqtfcpI Ugpi wrxc +"

Kvtf wekpí í í 0 í 0 í 047"

Y qtm' Ceeqo r nkuj gf í í 0 í 048"

!!

""Vcum'4d/6'I W'"LqtfcpUgpi wrx I0dkqtcj +'

Kvtafwepi í í 0 í 0 í 0005"

Y qtm'Ceego r rkuj gf í í 0 í 056"

!!

""Vcum'4d/7'*I W/"Lqtfcp10dkqtcj +"

Kvaf wekapi í í 0 í 0 í 006: "

[illegible]

!!

Vcum'5'''

""""Vcum5*I W/"Tkgi gnly gmwgkp+"'

[illegible]

Y qtm'Ceego r nkuj gf í í 0 í 082"

!!

Vcum'6'''

""""Vcum'6e'*VI gp'ô"C/ qtuc IDcxi wt wpcvj cp IEwprkkg+"'

Kotaf wekapí 96"

Vcdrg'qhEqpvgpw'èqpvkpwgf í 0'

"

Y qtnlCeeqo r nkuj gfí í í í í Œ í 07"

"

Mg{ 'Tgugctej 'Ceeqo r nkuj o gpwŒ í 00 4"

Tgr qt vcdrg'Qweqo guí 0000 9"

Eqpenwukppuí í í í Œ í 0 3"

TghgtgpeguŒ í í í Œ í 0 6"

Crr gpflzí í í í í Œ í 0 ;"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

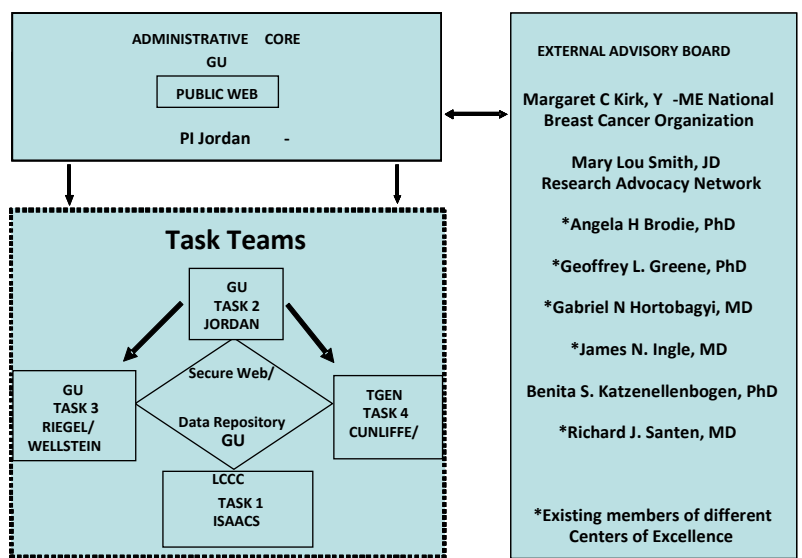
"

"

KP VT QF WE VKQP "

Vj g" Egpvgt" qh" Gzegmpep" I tcpv" ku" eqo r ngvpi " hqwt" kpf gr gpf gpv" kpvgtcqqpgvgtf " cpf " u{pgti kuke" vumu" vq" cej kxg" vj g" i qcn" cpf " cpuy gt" vj g" qxgtctej kpi " s wgvkqp< " vq" f kexxgt " vj g" o gej cpluo " qh'gustqi gp/kpf wegf 'dt gcuv'ecpegt 'egmbr qr vuku' bpf 'gucdrkuj 'vj g' bnpkcn' tlcnu' qh'uj qt v/vgt o " my " f qug' gustqi gp' vt gcwo gpv' vq' ecwug' cr qr vuku' lp' cpvj qt o qpg' t gukncpv' dt gcuv'ecpegt 0' Vq' cej kxg" vj g" i qcn" y g" j cf " gucdrkuj gf " cp' kpvgi tcvgt " qti cpl ckvap " Hli 03+ y kj " c" hkuv' eruu" cf xkuqt { " dqctf " vj cv' kpmu" erpkcn' tlcnu" *Vcuni'3+ y kj " rcdqtcvgt { " o qf gnu" cpf " o gej cpluo u" *Vcuni4+ f tqvggo leu" *Vcuni5+ cpf " i gpqo leu" *Vcuni6+ 0'

Hli wt g'3"



Hli wt g'30Qti cpl ckvap' qh' vj g' EQG0'

Eqo r ngvqp' qh' vj g' Tggt i cpl ckvap' qh' vj g' EQG'

Vj g" r cuv" { gct " j cu'uggp" c" f tco cve" kpetgcug" kp" qwt " tckpki " qh' pgy " uvelh' vj cv' j cu' cf f gf " vq" qwt " r tqf wvkvk { " cpf " i wctcpvgu" vj cv' y g" y km'o ckvap" qwt " tclgevt { " vq" gzt cpf " mpqy ngf i g" kp" vj ku" ko r qtcvp' pgy " ctgc' qh' y omen' u" j gcnj 0'

Qwt " y qtn' ku" j cxlpi " uki p hkecpv" ko r cev' kp" vj g' erpkcn' tlcnu" eqo o wplv { " y kj " vj g" tgegpv" r wdrkcvkqp" qh' vj g" Y omen' u" j gcnj " kpkcvkxg" uwf { " qh' gustqi gp' tgr mego gpv' vj gter { " cmppg" kp" j { uvgtevgqo k gf " y qo gp' vj cv' vj qy u' cp " *actual* " f getgcug" kp" vj g" kpek' gpeg" qh' dt gcuv' ecpegt " *3. " 4-0' Vj ku" gzeklpi " pgy " f gxmqr o gpv' kp" y qo gn' u" j gcnj " hpf u" ku" uelgv hke" hqwpf ckvap" kp" qwt " kppqxcvkg" i tcpv' cpf " r qlugf " vq" f ghkg" vj g" o gej cpluo u" pgeguvt { " vq" gzt mkl' gustqi gp' vj gter { " hwt vj gt " kp" vj g' erpkle 0' Vj g" y qtn' vj cv' y g" ctg' tghkpi " y km' hqto " vj g" dcuku" qh' cp' kpxkgf " ugtlgu" qh' tglxy u' qp" vj g" o qrgewrt " o gej cpluo " qh' gustqi gp/ kpf wegf " cr qr vuku 0' Vj tqwi j " vj g" cy ctf " qh' vj ku" Egpvgt" qh" Gzegmpep" I tcpv' hqo " vj g" F QF. " y g" j cxg" f go qpwtcvgt " kppqxcvqp" kp" uqrxkpi " hwpf co gpvnr tqdrgo u' kp" y qo gn' u" j gcnj " cv' vj g' o qrgewrt " hgxgr0'

"

DQF["

VCUM3<*NEEE Kcceu+/'Vq'eqpf wev'gzzr mjt cvqt { 'enplecn'v'kcn'vq'f gvgto kpg'vj g'ghlece{ "
cpf 'f qug't gur qpug'qh'r tq/cr qr vqle'ghgevu'qh'gum qi gp']Gum ceg 'kp'r cvlcpv'hmny kpi 'vj g'
hcnwt g'qh'v' q'lweguhwlc'p'vj qto qpcn'vj g'cr kgu0'"

Vcun3c<*Kcceu+/'Vq'eqpht o 'vj g'ghlece{ 'qh'wcpf ctf 'j k j 'f qug'gum qi gp'*Gum ceg+vj g'cr { "

cpf 'vj gp'f gvgto kpg'c'b kpk cnf qug'v'kpf weg'wo qt 'tgi tgukqp0'

J gtg'y g'tgr qtv'y qtn'eqo r ngvf "qp"Vcun3c"cv'vj g'NEEE"cpf "HEEE"ukgu"f wtkpi "{ gct"7"qh'vj ku"
 EQG0"

Enplecn'v'kcn'eqpf wevgf "d { 'Encw'kpg'Kcceu.'O F "

FQUG" FG/GUECNCVKQP" QH' GUVTQI GP" *GUVTCEG+" VQ" TGXGTUG"
 CPVK QTO QPG" TGUKVCPEG" KP" RCVKGPVU" CNTGCF[" GZJ CWUVKXGN["
 VTGCVGF 'Y KJ 'CPVK QTO QPG'VJ GTCR["

Y qtnCeeqo r kji gf <'

Kp"O c { "4233."Cwte"\ gpgec"grgevgf "vq'y kj ftcy "hwpf kpi "hqt"vj g'eqpf wev'qh'vj g'enplecn'
 v'kcn0"Cu'c'tguwn'qh'vj g'ncen'qh'hwpf kpi . 'ceetwcn'cv'vj g'Hqz'Ej cug'Ego r tgi gpukxg'Ecepgt'Egpgt"
 *HEEE+"cpf "qvj gt"chhkcvgf "ukgu"j cf "vq"dg"vgto kpcvgf 0""Kpukwkwpcn'hwpf u'y gtg'ugewt gf "hqt"
 eqpvkpwgf "ceetwcn'hqt"vj g'uwwf { "cv'vj g'Nqo dctf k'Ego r tgi gpukxg'Ecepgt'Egpgt"*NEEE+0""Chgt"
 ectghwn'eqpukf gtcvkqp." vj g' v'kcn' y cu' vj gtghqtg" r gto cpgpv { "enqugf " vq" ceetwcn' cv' HEEE" cpf "
 chhkcvgu" dw' tgo clpgf " qr gp" cv' NEEE0" Vj g' r tqveqn' y cu' v'cpukxkpgf " vq" NEEE" wpf gt" vj g"
 f kgevkqp'qh'Encw'kpg'Kcceu.'O F 0"C" r tqveqn'co gpf o gpv' y cu'wdo kwgf "vq"vj g'F gr ctwo gpv'qh"
 F ghpgug'v'q'tghgevg'vj g'ej cpi g'qh'RKcpf "enquwtg'vq'ceetwcn'cv'vj g'qvj gt'ukgu0"Chgt'F QF "cr r tqxcn"
 vj g'co gpf gf "r tqveqn' y cu'wdo kwgf "vq"vj g'KTD"cv'I gqti gvy p"Wpkxgtuk{ 0'F wtkpi "vj ku"r tqegu." "
 vj gtg'y cu'c"vgo r qtct { "eguvcvkp"kp"ceetwcn0"Qpeg"vj g'uwwf { "y cu'tguwo gf . "uetggkpi "dgi cp"cv"
 NEEE"cpf "3"r cvlcpv'j cu'dggp"ceetwgf 0"Uj g'ku'ewtgpvn{ "qp"y ggm: "qh'Gumceg"cpf "vqrgtcv'kpi "k"
 y gm0""Cf f kkpqm{ . 'F t0Tco qpc"Uy cd { "ngh'HEEE"cpf "F t0O ct { 'F cn{ "vgo r qtctkn{ "vqmiq'xgt"cu"
 RKqh'vj g'uwwf { "cv'HEEE0"Vj g'HEEE"ukg'ku'pqy "enqugf 0'

Qpg'qh'vj g'dcttktu'vq'ceetwcn'pqvgf "d { "HEEE"cpf "qvj gt'ukgu"j cf "vq'f q'y kj "vj g'f qulpi "qh"
 gumtcf kqr0""C"tgegpv"tcpf qo k gf "r j cug"Kkuwwf { "j cf "gzco kpgf "vj g'ko r cev'qh'my gt/f qug'xgtuwu"
 j k j gt/f qug'gumqi gp"vj g'cr { "kp"y qo gp"y kj "cf xcpegf "j qto qpg'tgegr vt"r qukkxg'dtgcuv'ecpgt"
 y j q'j cf "gxkf gpeg'qh'f kugcug'tgukxpeg"vq"ctqo cvcug'kpj kdkqtu"*5+0"Kp"vj ku'uwwf { . "vj g'enplecn'
 dgpghk'vcg'kp'dqy "i tqwr u'y gtg'pgctn{ "kf gpv'kcn'4: ' "kp'vj qug'tgegkxkpi "52"o i lf ckn{ "xgtuwu"4; ' "
 kp'vj qug'tgegkxkpi "8"o i lf ckn{ 0"J qy gxgt. "vj g'cf xgtug'gxgpv'tcvg"*@i tcf g"5+y cu'uki pkhcpcvn{ "
 my gt'kp'vj qug'tgegkxkpi "8"o i lf ckn{ *3: ' +cu'eqo r ctgf "vq"c"56' "tcvg'kp'vj qug'qp"52"o i lf ckn{ *r "
 ?"05+0""I kxgp"vj g'uko kct'dgpghk'vcg"y kj "dgwgt"vqrgtcpeg"r tqhkg."y g'grgevgf "vq"o qf kh{ "qwt"
 r tqveqn' vq"8"o i lf ckn{ 0" Vj ku'co gpf o gpv' ku'ewtgpvn{ "wpf gt"tgxkgy "cv'vj g'F QF "cpf "qpeg"
 cr r tqxgf "y kn'dg"uwo kwgf "vq"vj g'KTD"cv'I gqti gvy p"Wpkxgtuk{ 0""Qpeg"vj ku'ku'cr r tqxgf "y g"

r np"q"gzr cpf "qwt"ceetwcn"q"qyj gt "ukgu0"Y g"cnuq"r np"q"cf xgtvug"vj g"rtqvceqn"cpf "cpvlekr cvg"
vj cv."i kxgp"vj g"lpvgtgu"qh"r cvkcpw"cpf "vj gkt"ectgi kxgtu"lp"r tqmipi kpi "vj g"ghkece { "qh"gpq qetkpg"
vj gter { "cu"y gmi"cu"vj g"gzegmcpv"vqngtcdkkl { "qh"vj g"mry gt "f qug."vj cvceetwcn"y knlpetgcug0""

"

"

VCUM'4<*'I WLqtf cp+/'Vq'gnwelf cvg'vj g'o qngewrt 'o gei cpluo 'qhl'G4'*gum qi gp+'lpf weg f "
umt xkxcn'cpf "cr qr vquku'lp"dt gcu'ecpegt "egmi't gukucpv'vq'glvj gt "ugrgevkxg"GT'"*gum qi gp"
tgegr vqt +b qf wrcvqt u'UGTO u'qt 'mipi /vgt o 'gum qi gp'f gr t kxcvqp0'

Vcunl'4c<*"Ctk| k'cpf 'Lqtf cp+/'Vq'eqo r ngvg'c'ugt lgu'qhl'gzr gt ko gpw'wulpi 'ugw'qhl'y gmi'
f ghlpf 'dt gcu'ecpegt 'b qf gnu'qhl'G4/lpf weg f 'umt xkxcn'cpf 'cr qr vquku' *in vivo* cpf ' *in vitro*]cv'
vj g'HEEE_0'HEEE'y knl'i gpgt cvg'r tqvglp'uco r ngu'hqt 'rt qvgqo le'cpcn[ugu]ectt lgf "qww'
wpf gt 'Vcunl'5']cvI W'cpf 'TPC'uco r ngu'hqt 'i gpg'gzr t gukqp'b let qcttc { 'cpcn[ugu]ectt lgf "
qww'wpf gt 'Vcunl'6']cvVt cpur vqpncil gpqo ku'T gugetej 'kpukswwg'*VI gp+0''

Gum qi gp/lpf wegu'Cr qr vquku'lp"Gum qi gp" F gr t kxcvqp/t gukucpv'Dt gcu'Ecpegt "Vj tqwi j "
Ut guu'T gur qpugu'cu'k gpvhlgf 'd { 'I mdcnI gpg'Gzr t gukqp'Cet quu'Vlo g0'

Y qtmiCeeqo r ikuj gf <'

Vj g"fcv"cpf "cpcn[uku"eqo r ngvg'f wtkpi "vj g" { gct'hqt"vj ku'vcunl'ctg"uwo o ctk gf "lp"vj g"r wdrkcvkp<
Ctk| k'G0C0'Ewprkhhg."J G0'Ngy ku/Y co dk'L0U0'Ukngt."O L0'Y knku."C0N0'Tco qu."R0"Vcr kc."E0"
Mko ."J 0T0' [gttwo ."U0'Uj cto c."E0 (P0'P leqrnu."G0'Dcni wt wpcvj cp."I 0'Tquu."G0C0'cpf "Lqtf cp."
XŒ0' *4233+0' Gumqi gp/lpf wegu" Cr qr vquku' lp" Gumqi gp" F gr t kxcvqp/t gukucpv' Dt gcu' Ecpegt "
Vj tqwi j "Ut guu'T gur qpugu'cu'k gpvhlgf 'd { 'I mdcnI gpg'Gzr t gukqp'Cet quu'Vlo g0Rt qeggf kpi u'qhl'
vj g" *Pcvkqpcn'Cecf go { "qhl'Uekpegu" WUUC0'32: -3: : 9; /3: : : 8"*ugg'0'lp'Cr r gpf lz +0'Vj gug'fcv"*
ugt xg"cu"qwt"r tko ct { "f cwcducg"ht"cm'uwdugs wgpv'uwf lgu"vq"dg"eqpf wevgf "f wtkpi "qwt"pq"equv"
gz vgpukqp0'

"

"

VCUM'4<*'I WLqtf cp+/'Vq'gnwelf cvg'vj g'b qngewrt 'b gei cpluo 'qhl'G4'lpf weg f 'umt xkxcn'cpf "
cr qr vquku'lp"dt gcu'ecpegt "egmi't gukucpv'vq'glvj gt "ugrgevkxg"GT'"o qf wrcvqt u'UGTO u'qt "
mipi /vgt o 'gum qi gp'f gr t kxcvqp0'

Vcunl'4d/3<*"Hcp'cpf 'Lqtf cp+'b'Vq'lp xguu' cvg'vj g'hpewkp'qhl'vj g'v' t qukpg'hlpucg'e/Ut e'lp"
G4/lpf weg f 'dt gcu'ecpegt 'egmi'umt xkxcn'cpf 'cr qr vquku'0''

Vcunl'4d/3<*"Hcp"cpf "Lqtf cp+/'Uwf lgu'ectt lgf "qww'd { "F t0'Rkpi "Hcp"lp"vj g" Lqtf cp"rdqtcvqt { "cv"
I gqti gvqy p"Wpkxgtukv' "

O qf wrcvpi "Vj gter gwle'Ghgevu'qhl'e/Ut e'kpi klsqt 'xlc'Gum qi gp'Tgegr vqt "cpf "J GT4'lp"
Dt gcu'Ecpegt 'EgniNlpgu'

"

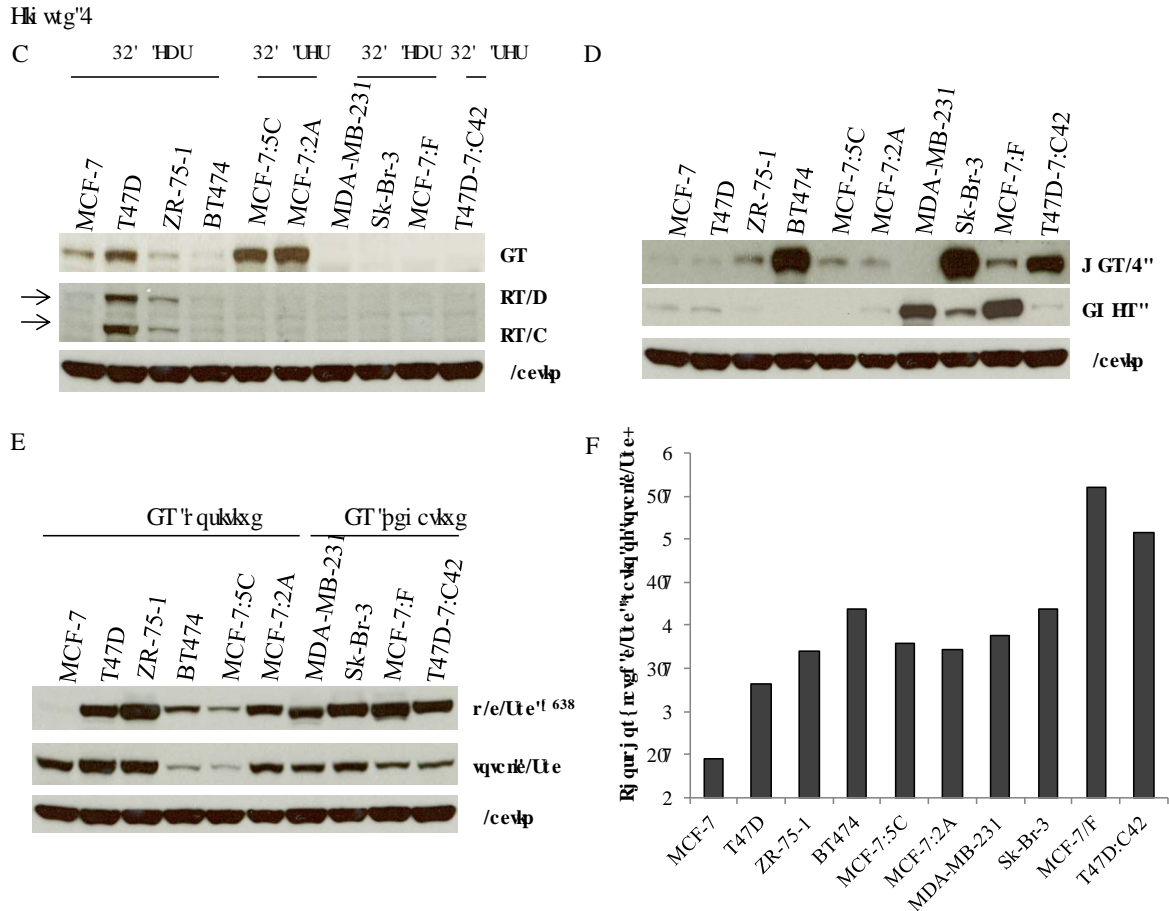
Kpvt qf wevkqp<"

Gutqi gp"tgegr vqt"*GT+"cpf"j wo cp"gr kf gto cni tqy vj "hcevt"tgegr vqt"4"*J GT4+"ctg"vy q" uweeguhwn"vj gter gwle"vcti guv"lp"dtgcu"ecpegt0'e/Ute"hwpevkpu"cu"cp"ko r qtcvpv"cf cr vgt"r tqvklp" y kj "GT"cpf"J GT4."y j lej "xcrkf cvgu"kv"cu"cp"cwtevkxg"vcti gv"ht"vj g"tgcv gpv"qh"dtgcu"ecpegt0' J qy gxgt."c"xgt{"tgegpv'r j cug"KKerplecni'vkcni'uj qy u"vj cv"e/Ute"lpj kdkqt."f cucvklp."j cf "rko ksf" ulpi ng/ci gpv"cevkxkv"lp"r cvkpvu"y kj "J GT4- "cpf lqt"j qto qpg"tgegr vqt"*J T+ "cf xcpegf"dtgcu" ecpegt"*6+0'Vj gtghqtg."c"ur gekhe"e/Ute"lpj kdkqt."RR4."y cu"wkkl gf"vq"dmqeni'e/Ute"cevkxkv"vq" kf gpvkl{"vcti gv"gf"xpwtgtdkklkv"chgevgf"d{"GT"cpf"J GT4"lp"e"r cpni'qh"dtgcu"ecpegt"egm'rkpgu" *Q0'OE9."V69F."T/97/3."DV696."OE9-7E."OE9-4C."OFC/OD/453."Umdt/5."OE9/ 9IH"cpf"V69F-64+0'Vj g"cpvkr tqnhtgcvkxg"ghgevg"qh"RR4"eqttgcv"y kj "dmqenpi"e/Ute"o gf kv"gf" GTMIO CRM" cpf lqt" RSMICm" i tqy vj " r cvj y c{u0' Vj g" uki pcnpi " r cvj y c{u" qh" e/Ute" y gtg" o qf wcv"gf" d{" GT" cpf " J GT4" lp" dtgcu" ecpegt" egm0' Kpj kdkkp" qh" e/Ute" v{tqulpg" nkpug" r tgf qo kpcpv"dmqengf"GT"pgi cvkxg"dtgcu"ecpegt"egm'i tqy vj ."r ctvewrtn{"vj g"vkr ng"*Q0'GT." r tqi guvgtqpg"tgegr vqt"*RT+"cpf"J GT4+"pgi cvkxg"egm0'Kp"eqpvcu."GT"pgi cvkxg"Umdt/5"egm" y kj "j ki j guv"J GT4" r j qur j qt{ncvqp"y gtg"tgukncpv"vq"RR4."lp"y j lej "qxgt/cevkxcvgf"J GT4" f kgevn{"tgi wcv"gf"i tqy vj "r cvj y c{u"dw"pqv"vj tqvi j "e/Ute0'J qy gxgt."dmqenpi"e/Ute"tgeqxtgf" GT"gzr tguukp"y j lej "o cf g"Umdt/5"egm"tgi clp"tgur qpugu"vq"6/j {ftqz{vco qzklp0'Vj g-o clqtkv{" qh"GT"r qukvxg"dtgcu"ecpegt"egm"y gtg"pqv'ugpvukxg"vq"RR4"tgi ctf ngu"qh'y kf/v{r g"qt"gpqf qetlp" tgukncpv'egm'rkpgu0'Qxgtcm'e/Ute"o gf kv"gu"vj g"guugpvkn'tqng"qh'i tqy vj "r cvj y c{u"lp"GT"pgi cvkxg" dtgcu"ecpegt"egm"y kj qw"J GT4"qxgt/cevkxcvqp0'GT"gzr tguukp"cpf"J GT4"qxgt/cevkxcvqp"btg" wy q"ko r qtcvpv"r tgf kvkxg"dkqo ctngtu"ht"vj g"tgukncpeg"vq"e/Ute"lpj kdkqt0'Vj gug"f cv"r tqxkf gf" cp"ko r qtcvpv"vj gter gwle"tcvqpcng"ht"r cvkpv'ugrgevkp"lp"erplecni'vkcni"y kj "e/Ute"lpj kdkqtu"lp" vkr ng"pgi cvkxg"dtgcu"ecpegt0'

Y qtmiCeego r rkuj gf<'

Dcuglpg'lgxgn'qh'GT.'J GT4.'cpf 'e/Ute'cevkxcvqp'lp'e'r cpni'qh'dtgcu'ecpegt 'egm'rkpgu'

Y g"cf f tguugf"vj g"s wgvkqp"y j gvj gt"gzr tguukp"qh"GT"cpf"i tqy vj "hcevt"tgegr vqtu"y qwf" chgevg"vj g"vj gter gwle"ghgevg"qh"vj g"e/Ute"lpj kdkqtu"lp"dtgcu"ecpegt"egm0'Vq"cpuy gt"vj ku" s wgvkqp."c"r cpni'qh'y kf/v{r g"*OE9."V69F."T/97/3."DV696."OFC/OD/453."cpf"Umdt/5+" cpf"gpqf qetlp"tgukncpv"*OE9-7E."OE9-4C."OE9/9IH"cpf"V69F-64+"dtgcu"ecpegt"egm' rkpgu"y gtg"lpvguki cvgf0'Dcuglpg"ngxgn'qh'GT."J GT4."GI HT."cpf"e/Ute"y gtg"o gcuwgf"d{" ko o wpqdmv'cpcn{uku0'Vj g{"cm'nggr"vj gk"dkqni keni'ej ctcevgtku"y kj "f khtgvpkn'lgxgn'qh'GT." RT."J GT4."cpf"GI HT"*Hki 04C"cpf"4D+0'Cm'egm'rkpgu"gzr tguugf"f gvgevdng'ngxgn'qh'vqcn'e/Ute." y j gtgcu"vj g{"o cplguvgf"f khtgvpv'lgxgn'qh'r j qur j qt{ncv"e/Ute"*Hki 04E+0'Cnj qwi j "vj gtg"ku"pq" enget"tgnvqpuij kr "dgvy ggp"e/Ute"r j qur j qt{ncvqp"cpf"J T"gzr tguukp"*Hki 04F+"chgt"pqto crk gf" d{"vqcn'e/Ute"co qpi "vgvgf"egm'rkpgu."lpvgtgukpi n{."y g"qdugtxg"vj cv'e/Ute"ku"cevkxcvgf"lp"tgukncpv" egm'rkpgu"eqo r ctgf"y kj "tgur gevkxg"r ctgpvni'egm'rkpgu"*OE9-7E."OE9-4C."cpf"OE9/9IH' xgtuwu"OE9."V69F-64"xgtuwu"V69F+0'

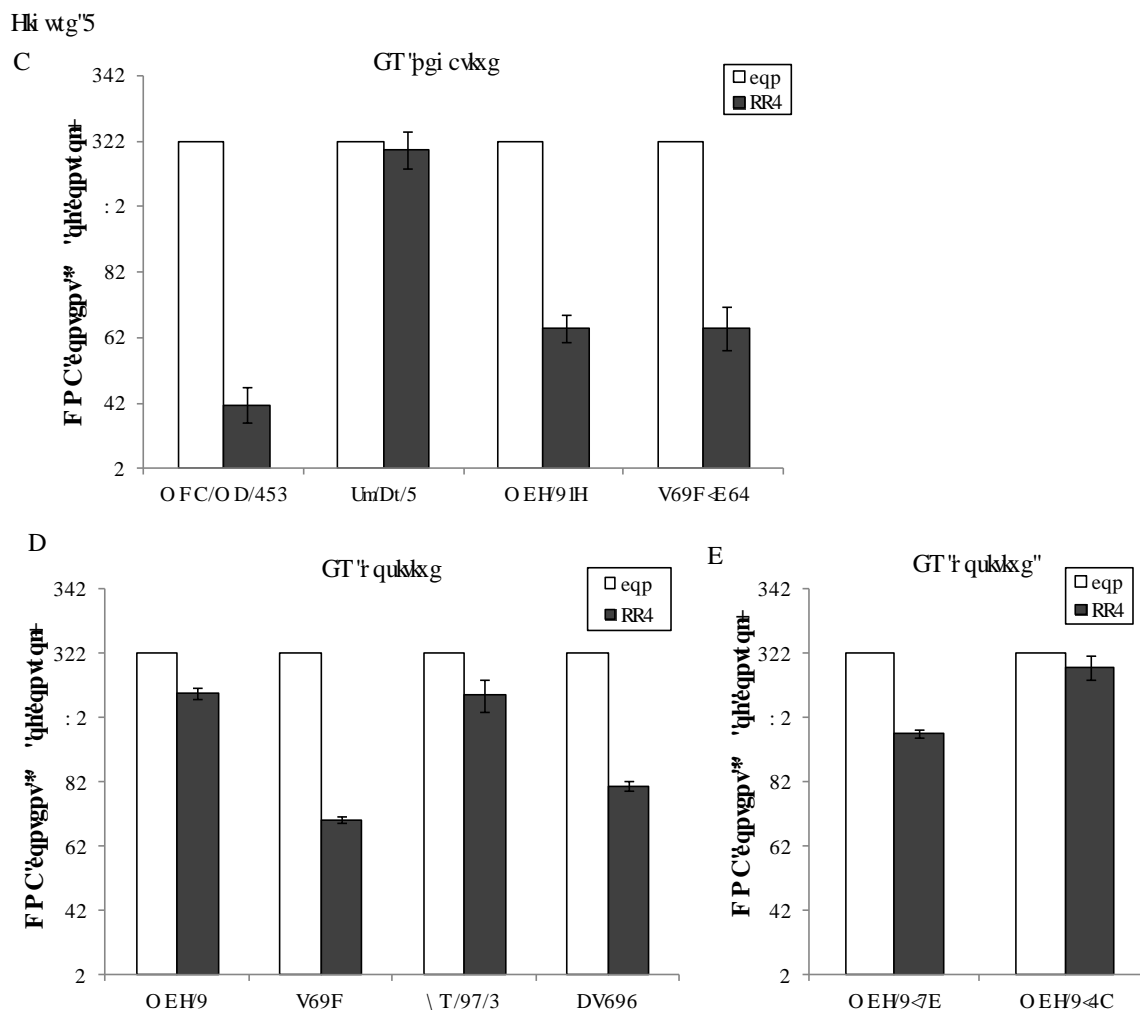


Hk wtg"40Dcuglpg'gzzrtgukqp'qhlj qt o qpg'tgegrvqtu'cpf 'i tqy vj 'hcevt'tgegrvqtu'lp'f Hhgt gpv'egmihpgu'4C0'
Dcuglpg'GT 'cpf 'RT'gzzrtgukqp'igxgn'lp'f Hhgt gpv'egmihpgu'0EH/9."V69F." T/97/3."DV696."OFC/O/D/453."
 UnlDt/5."cpf "OEH/9IH'egm' y gtg'ewwtgf "kp" gxtqi gpk gf "o gf kwo "32' "HDU-0'OEH/9<E."OEH/9<C."cpf "
 V69F <E64'egm' y gtg'ewwtgf "kp" rj gpqnl'tgf "htg" o gf kwo "eqpcklpi "ej cteqcn'utkr r gf "ugt wo "32' "UHU-0'Egm'
 n'ucvgy gtg"j ctxgugf 0GT "cpf "RT" gzzrtgukqp'igxgn' y gtg'gzco kpgf "d{ "ko o wpqdmwpi "y kj "r tko ct { "cpvdkf lgu'
 ko o wpqdmwpi "hqt" /cevp" y cu" f gvgto kpgf "hqt" mcf lpi "eqpvtqn'04D. **Dcuglpg'J GT4'cpf 'GI HT'gzzrtgukqp'igxgn'**
lp'f Hhgt gpv'egmihpgu'0Egm'n'ucvgy gtg"j ctxgugf "cu"cdqsg0J GT4"cpf "GI HT" gzzrtgukqp'igxgn' y gtg'gzco kpgf "d{ "
 ko o wpqdmwpi "y kj "r tko ct { "cpvdkf lgu' ko o wpqdmwpi "hqt" /cevp" y cu" f gvgto kpgf "hqt" mcf lpi "eqpvtqn'04E.
Dcuglpg'e/Ute'r j qur j qt { r/vqcnle/Ute "r/vqcnle/Ute + **Egm'n'ucvgy gtg"j ctxgugf "cu"cdqsg0Rj qur j qt { r/vgf "e/Ute"**
 cpf "vqcnle/Ute" y gtg' f gvgvgf "d{ "ko o wpqdmwpi "y kj "r tko ct { "cpvdkf lgu' ko o wpqdmwpi "hqt" /cevp" y cu" wugf "hqt"
 mcf lpi "eqpvtqn'04F. **S wcpvllcckp'qhlj j qur j qt { r/vgf 'e/Ute'd{ "vqcnle/Ute0Rj qur j qt { r/vgf "e/Ute"lp'f Hhgt gpv'egm'**
 n'pgu' y cu's wcpvllcckp' d{ "j g'vqcnle/Ute" wupi "S wcpvllcckp' "Qpg"uqhy ctg'htqo "Dkq/Tcf 0'

Vj g'e/Ute'lpj kdkqt 'ghgevkxg' 'dmqengf 'GT'pgi cvkxg'dt gcu'ecpegt 'egmli tqy vj 0'

Vj g'cpw'r tqnlhgtcvkxg'ghgevkxg'qhlRR4'eqttnrvgf "y kj "dmqenlpi "e/Ute" o gf kcvgf "GT MIO CRM"
 cpf lqt "RK MICm" i tqy vj "r cyj y c { u0Vj g'uki pcnpi "r cyj y c { u"qh'e/Ute" y gtg" o qf wrcvgf "d{ "GT" cpf "
 J GT4"lp'dt gcu'ecpegt "egm'0Vj g" i tqy vj "lpj kdkqt { "ghgevkxg" d{ "j g'e/Ute'lpj kdkqt "qp"GT"r qukxg"
 egm'crr gct "vq"dg" o qtg"eqo r ngz "vj cp"qp"GT"pgi cvkxg"egm'0lpj kdkqp"qh'e/Ute"v{ tqukpg"n'kpcug"
 r tgf qo kpcpwl "dmqengf "GT"pgi cvkxg"dt gcu'ecpegt "egmli tqy vj . "r ct vkwrcn { "vj g'vkr ng"04G0GT. "RT."

cpf "J GT4+"pgi cɤxg"egmɪ***H**ki 0'5C+0'Vj g-o clqtɤv{ "qh"GT"r qukkɤg"dtgcu'vecepgt"egmɪ'y gtg"pqv"
ugpukkɤg"vq"RR4"tgi ctfguu"qh'y kf/vj r g"qt"gpq qetɤpg"tgukɤcpv"egmɪ'ɤpgu"***H**ki 0'5D"cpf "5E+0'kɤ"
eqpɤcu."GT"pgi cɤxg"UmDt/5"egmɪ'y kj "j ki j guv"J GT4"rj qur j qt{rɤvɤp"y gtg"tgukɤcpv"vq"RR4"
***H**ki 05D+."kɤ'y j ke j "J GT4"ftgeɤv{ "tgi wɤvɤf "i tqv vj "r cɤv y c{u'dw'pqvɤ tqwi j "e/Ute0'



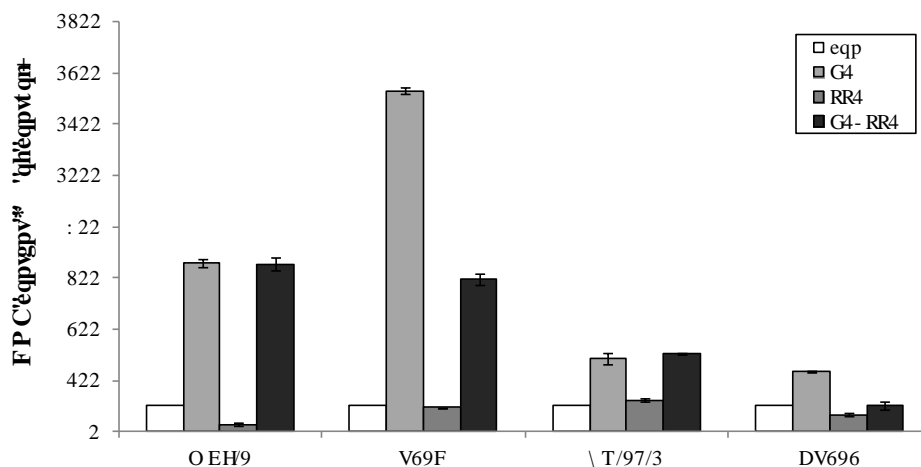
Hli wtg'50I t qy vj 'lpj klsqt { 'ghgewu'qh'vj g'e/Ute'lpj klsqt "qp" c"r cpgn'qh'dtgcw'ecpegt 'egmu,5C0I t qy vj "
lpj klsqt { 'ghgewu'qhRR4'lp'GT'pgi cvkxg'egmuGT'pgi cvkxg'egmu'y gtg'uggf gf 'lp'46/y gmi'r rvgu'lp'vtr necvgO'Chgt "
 qpq'f c{. "vj g'egmu'y gtg'v'gcvgf "y kj "xgj kerg"*2Ø' F O UQ+cpf "RR4"*7 O +lp'32' "UHU'o gf kwo 0'Vj g'egmu'y gtg"
 j ctxgugf "chgt"9"f c{u'v'gtcvo gpv'cpf "vqcn'F P C"y cu'f vgtgo kpgf "wulpi "c'F P C"hwqtguepgpeg's wcpvkcvkp"nksO'**5D0'**
I t qy vj 'lpj klsqt { 'ghgewu'qhRR4'lp'GT'r quksxg'y kf /v/r g'egmuO'Y kf /v/r g'GT'r quksxg'egmu'y gtg'uggf gf 'lp'46/
 y gmi'r rvgu' lp" vtr necvgO' Chgt" qpq'f c{. "vj g'egmu" y gtg" v'gcvgf " y kj " xgj kerg" *2Ø' F O UQ+" cpf " RR4" *7 O +"
 t g u r g e v k x g n (0'Vj g'egmu'y gtg"j ctxgugf "chgt"9"f c{u'v'gtcvo gpv'cpf "vqcn'F P C"y cu'f vgtgo kpgf "cu'cdqxgO'**5E0I t qy vj "**
lpj klsqt { 'ghgewu'qhRR4'qp'GT'r quksxg'gpf qet lpg't gukwcpv'egmi'lpguO'OE H'9>7E" cpf "OE H'9>4C"egmu'y gtg"
 uggf gf "lp'46/y gmi'r rvgu'lp"vtr necvgO'Chgt"qpq'f c{. "vj g'egmu'y gtg'v'gcvgf "y kj "xgj kerg"*2Ø' " F O UQ+cpf "RR4"
 *7 O +t g u r g e v k x g n " lp" ewnwgtg" o gf kwo 0' Vj g'egmu'y gtg"j ctxgugf " chgt"9" f c{u' v'gtcvo gpv' cpf " vqcn' F P C" y cu'
 f vgtgo kpgf "cu'cdqxgO"

Vj g'e/Ute'ehgevgf 'vj g'hwpevkqp'qh'GT'lp'GT't qukskg'egm0'

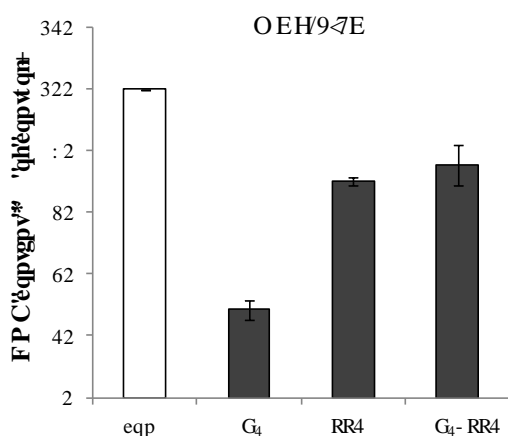
Ukpeg"gutqi gp"uko wrvgu"vj g"i tqy vj "qh'GT'r qukskg'y kf/v{r g"dtgcu'ecpegt"egm."hwt" GT'r qukskg'y kf/v{r g"dtgcu'ecpegt"egm"y gtg'uko wrvgf "d{ "gutqi gp"*G4+'q"i tqy "y kj "f khtgtpv" ugpukxkv{ "*Hki 0'6C+0"P qvcdn{ ."RR4"eqwrf "pqv'dmjen'vj g'r tqrkhtgcvkqp"lpf wegf "d{ "G4"lp"O EH/9" cpf "\ T/97/3"egm"dw'r ctvkm{ "cdqrkuj gf "G4"uko wrvkqp"lp"V69F "cpf "DV696"egm"*Hki 0'6C+0' Vj gug"tguwu"lpf kcvgf "vj cv'e/Ute"o ki j v'r rc{ "c"fkvpev"tqrg"lp"o gfkvpi "G4"uki pcrpi "lp"y kf / v{r g"egm0'lp"y q"gpqetkpg"tgukvcpv"egm"*O EH/9<7E"cpf "O EH/9<4C+."vj cv'qxgtgzr tguu"GT." RR4"eqwrf "dmjen'e/Ute"cevkcvkqp"cpf "cdqrkuj gf "cdqw'47' "qh'r tqrkhtgcvkqp"lp"O EH/9<7E"egm." dw"y kj qw"cp{ "lpj kdkkqp"lp"O EH/9<4C"egm"*Hki 0'5E+0'Qwt"r tgxkqwu"fcv"uj qy gf "vj cv'G4"j cu" vj gter gwle"hwpevkqp"vq"lpf wegf "cr qr vuku"lp"npi /vgt "G4"fgt kxgf "dtgcu'ecpegt"egm0'Y g" tgcupgf "vj cv'e"eqo dlpvcvqp"qh"RR4"y kj "G4"y qwrf "gpj cpeg"G4/lpf wegf "cr qr vuku0'Uwrtkulpi n{." RR4"f kf "pqv'gpj cpeg"vj g"i tqy vj "lpj kdkqt{ "ghgew"qh'G4"qp"vj gug"vy q"egm'rkpgu."dw'dmjenf "vj g" i tqy vj "lpj kdkkqp"lpf wegf "d{ "G4"*Hki 0'6D"cpf "6E+0'Vj gug"fcv"ko r rkf "vj cv'G4/vtki i gtgf "cr qr vuku" o ki j v'dg"wkklpi "e/Ute"v{tqukpg"mkpcug"cu"cp"ko r qtvcvpi uki pcrpi "rcvj y c{0'Y g"ctg"ewtgpv{ " kpxgunki cvpi "vj g"o gej cpluo u"qh"j qy "vj g'e/Ute"lpj kdkqt"dmjen"G4/vtki i gtgf "cr qr vuku0'Y g"y km' uj qy "tgrvgf "fcv"lp"vj g'hmqy lpi 'r ct0'

Hk wt g'6

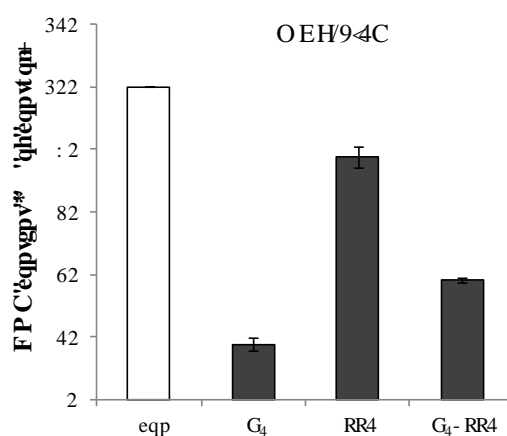
C



D



E



Hk wt g'60 6C0Vj g'RR4'j cf 'f hgt gpv'ghgew'qp'G4'wko wr vkw'lp'GT'r quksxg'y kf /v{ r g'egm0'Y kf /v{ r g'GT' r quksxg'egm'y gtg'ej cpi gf "v"G4'ltgg'o gf kwo "hqt"5"f c{ u0Vj gp. "vj g{ "y gtg'uggf gf "lp"46/y gnr'rvgu0Chgt"qpg"f c{. " y g'egm'y gtg'vgtg'f y kj "xgj lerg"20' "GvQJ +."G4"*32/o qnlN+: "RR4"*7 O +."cpf "G4"*32/o qnlN+: "rru"RR4"*7 O +." tgr gevxgn{ "lp" G4'ltgg'ewwtg" o gf kwo 0' Vj g' egm'y gtg'j ctgugvf "chgt"9"f c{ u'vgtcvo gpv'cpf "vqcn'F P C" y cu' f gvtgto lpgf "cu'cdqxs06D0Vj g'RR4'ltqengf 'G4/lpf wegf 'lpj klskqp'lp'O EH/9-7E0'EH/9-7E'egm'y gtg'uggf gf "lp" 46/y gnr'rvgu'cu'cdqxs0Chgt"qpg"f c{. "y g'egm'y gtg'vgtg'f y kj "xgj lerg"20' "GvQJ +."G4"*32/o qnlN+: "RR4"*7 O +." cpf "G4"*32/o qnlN+: "rru"RR4"*7 O +."tgr gevxgn{ 0Vj g'egm'y gtg'j ctgugvf "chgt"9"f c{ u'vgtcvo gpv'cpf "vqcn'F P C" y cu' f gvtgto lpgf "cu'cdqxs06E0Vj g'RR4'ltqengf 'G4/lpf wegf 'lpj klskqp'lp'O EH/9-4C0'EH/9-4C'egm'y gtg'uggf gf "lp" 8/y gnr'rvgu0Chgt"qpg"f c{. "y g'egm'y gtg'wko krtn{ "vgtcvo "cu'lp'O EH/9-7E'egm0Vj g'egm'y gtg'j ctgugvf "chgt"36" f c{ u'vgtcvo gpv'cpf "vqcn'F P C" y cu'f gvtgto lpgf "cu'cdqxs0'

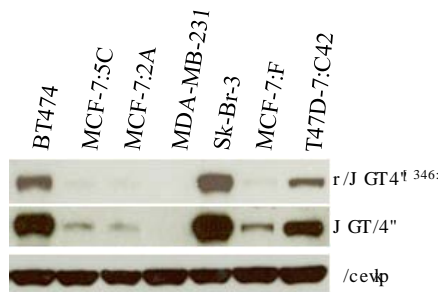
Cevkxcvkw'lwvuw'qhJ GT4'f gvtgto lpgf 'vj g'lpj klskqp { 'ghgew'qh'vj g'e/Ute'lpj klskqp 0'

J GT4"qxgtgzt tguakp" ncf u"v" c" xgt { "ci i tguakxg"ecpegt" rj gpqv{ r g" cpf "r qqt" r cvkpv' uwtxkcn' *7-Œ/Ute" ku" npqy p" v" dlpf "v" J GT4" cpf "ku" y wu'cevkwcvf "lp" J GT4/qxgtgzt tguakpi " ecpegt"egm0DV696"cpf "UwDt/5"egm'qxgtgzt tguu"gpqi gpqwu"J GT4"*Hk 07C+."j qy gxgt. "y g{ " j cf "f hgt gpv'tgr qpugu"v"RR4"*Hk 05C"cpf "5D-0Vq"gzco lpg"y j gj gt"J GT4"cevkwcvkw'chgew' yj g'lpj klskqp { "tcvg'qh'RR4." rj qur j qt { rkw'qh'J GT4'y cu'gxcnvcv 0'J GT4'y cu'j ki j n{ "cevkwcvf "

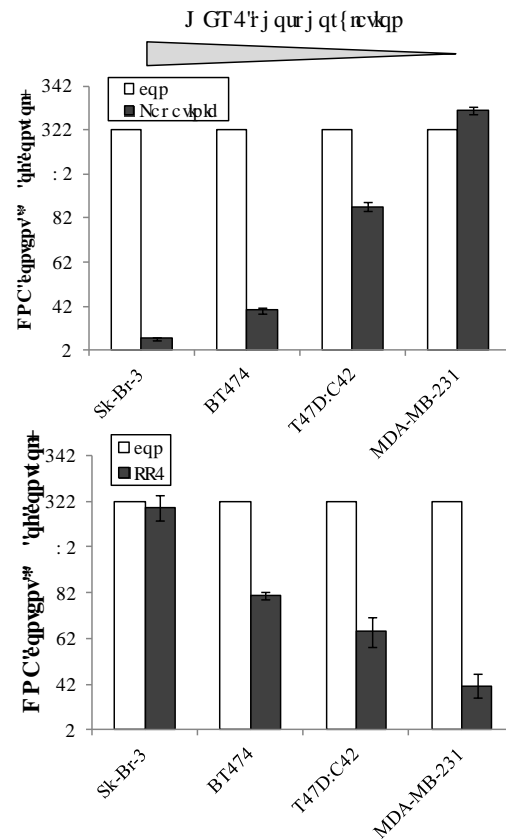
lp"UmDt/5"egmu"y j lej "o cf g"kl"j {r gtugpukxg"vq"ncr cvlpld."c"fwcn"v{tqulpg"nlpcug"lpj kdkqt"qh" J GT4"cpf"GI HI"Hi 07C"cpf"7D+0Vj g"i tqy vj "lpj kdkqt{"ghgevu"d{"ncr cvlpld"eqttgur qpf gf"vq" vj g"lrgxgm"qh"rj qur j qt {ncvlf"J GT4"Hi 07C+0Y g"qdugtugf"vj cvJ GT4"j {r gt/cevxcvlp"tgpfgtgf" dtgcu"ecpegt"egmu"eqo r nvgm{"tgukucpv"vq"RR4="vj g"j ki j gt"J GT"rj qur j qt {ncvlp."vj g"mgy gt" tgur qpukxg"tcvg"vq"RR4"Hi 07D+0Vj ku"y cu"lwtvj gt"eqphko gf"d{"U"rj cug"ej cpi gu"vj tqwi j "lmqy " e{vqo gytke" cpcn{uku" Hi 0' 7E+0' Ncr cvlpld" tgf wvgf" U" rj cug" lp" egmu" y kj " j ki j gt" J GT4" rj qur j qt {ncvlp."eqpxgtugn{."RR4"y cu"ghgevkxg"lp"egmu"y kj "mgy gt"J GT4"rj qur j qt {ncvlp"Hi 0' 7E+0

Hi wtg'7

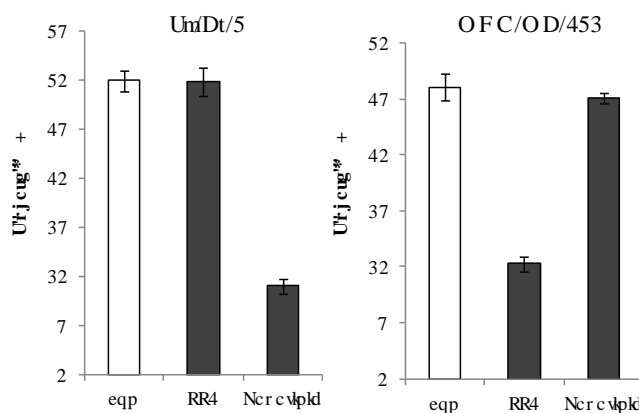
C



D



E



Hi wtg'70'Cevxcvlp"ucwvu"qh"J GT4"i gvgto lpgf"vj g"lpj kdkqt{"ghgevu"qh"vj g'e/Ute"lpj kdkqt"07C0'Dcuglpg" J GT4"rj qur j qt {ncvlp"lp"i lhtgtpv'egm"lpgu"0'Egm"n{ucvgu"y gtg"j ctxgugf"ltqo "f lhtgtpv'egm"0'Rj qur j qt {ncvlf" J GT4"cpf"vqcn"J GT4"y gtg"gzco lpgf"d{"ko o wpqdmwlp"i"y kj "r tko ct{"cpvldqf lgu"0'ko o wpqdmwlp"i"lht" /cevlp"y cu" f gvgto lpgf"lht"mcf lpi "eqptqir"0'7D0'lpj kdkqt{"ghgevu"qh"vj g"J GT4"lpj kdkqt"cpf"vj g'e/Ute"lpj kdkqt"qp"egm"i" y kj "lrgxcvgf"J GT4"rj qur j qt {ncvlp"UmDt/5."DV696."V69F-E64."cpf"OFC/OD/453"egm"y gtg"uggf gf"lp"46/ y gm"r ncvgu"lp"vtr ldecvg"0'Chgt"qpq"fc{"vj g"egm"y gtg"vtgcvgf"y kj "xgj lerg"*208' FO UQ+."ncr cvlpld"*3 O+."cpf"RR4" *7 O+"lp"32' "UHU"o gf kwo 0'Vj g"egm"y gtg"j ctxgugf"chgt"9"fc{u"vtgcvo gpv"cpf"vqcn"FO UQ"y cu"fgvgto lpgf"cu" cdqxg"0'7E0'U"rj cug"ej cpi gu"chgt"ncr cvlpld"cpf"RR4"vtgcvo gpv"0'UmDt/5"cpf"OFC/OD/453"egm"y gtg"vtgcvgf" y kj "xgj lerg"*208' "FO UQ+."ncr cvlpld"*3 O+."cpf"RR4"*7 O+"lht"46j 0'Egm"y gtg"j ctxgugf"cpf"lhzgf"y kj "97' " GxQJ 0'Egm"n{ergu"y gtg"cpn{| gf"vj tqwi j "lmqy "e{vqo gyt{0"

Qxgtcm'e/Ute"o gf kcvu"vj g"guugpvkn'tqrg"qh'i tqy vj "rcvj y c{u"lp"GT"pgi cvkxg"dtgcu' ecpegt"egm'y kj qw"J GT4"qxgt/cevxcvqp0'GT"gzrtguukqp"cpf "J GT4"qxgt/cevxcvqp"ctg"vy q" ko r qtcvpv'r tgf kevkg"dkqo ctngtu"ht"vj g"tgukxpepg"vq"e/Ute"lpj kdkqt0'Vj gug"fcv"r tqxkf gf "cp" ko r qtcvpv'vj gter gwke'tcvkpcrg'ht'r cvkpv'ugngevqp"lp'enplecn'vkn'y kj 'e/Ute"lpj kdkqtu'lp'vtr ng" pgi cvkxg"dtgcu'ecpegt0Cn'qh'vj gug"fcv"y kn'dg'r vdrkuj gf "lp"vj g"Gwtqr gcp'Lqwtpcn'qh'Ecpegt"p" r t g u u +=l u g g "%&: 'lp'Cr r gpf kz'ht'r tqghu0'

"

"

VCUM4<"*1 Wlqtf cp+/'Vq'gwelf cvg'vj g'b qngewrt'b gei cpluo 'qh'G4'lpf wegf 'lwt xkxclbpf "
cr qr vquk'lp'dt gcu'ecpegt 'egm't gukxcpv'vq'glxj gt 'ugngevkg'GT'o qf wrcvqt u'*UGTO u'qt "
mipi /vgt o 'gwt qi gp'f gr t kxcvqp0'

Vcun'4d/4<"*Hcp"cpf "Lqtf cp+'ó"Vq'eqphkto "cpf "xclb cvg'f gxgnr lpi "rcvj y c{u'qh'G4/
lpf wegf 'dt gcu'ecpegt 'egniltwt xkxclbpf 'cr qr vquk0'

Vcun'4d/4<"*Hcp"cpf "Lqtf cp+/'Uwf lgu"ecttkgf "qw'd{ "F t0'Rkpi "Hcp"lp"vj g"Lqtf cp"rdqtcvqt {"cv"
I gqti gvqy p"Wpkgtukf }

Et kkecn0 gf kcvqp'qh'G4/lpf wegf 'Cr qr vquk'vj tqwi j 'e/Ute'lp'Nqpi /vgt o 'Gwt qi gp'F gr t kxgf "
Dt gcu'Ecpegt 'Egm'

Kpvt qf wekqp<'

Qwt"rtgxkqu"r vdrkecvqp"uj qy gf "vj cv'r j {ukmqi kecn'eqpegpvcvqp"qh'G4"eqwrf "vki i gt" cr qr vquk'qh'mipi /vgt o "G4"f gr t kxgf "dtgcu'ecpegt"egm"*O EH/9<E+0'Vj ku'pgy "vcti gvgf "utcvgi {" r tqxkf gu'pqxgn'vj gter gwke"cr r tqcej gu"vq"gpf qetkpg"tgukxcpv'dtgcu'ecpegt0'C"r j sug"K'Enplecn' vkn'tgr qtvgf "vj cv'G4"r tqxkf gf "c'enplecn'dgpgkv'ht"ctqo cvcug'lpj kdkqt/tgukxcpv'cf xcpegf "dtgcu' ecpegt'r cvkpw0J qy gxgt."qpn{"52' "qh'r cvkpw'tgekgxg'enplecn'dgpgkv"*5+0'Vj ku'r tqo r vgf "wu"vq" lpxguk cvg'vj g"o gei cpluo u'wpf gtn' lpi "G4/lpf wegf "cr qr vquk"vq"lpf "utcvgi lgu"vq"lpetgcug"vj g" vj gter gwke'tgur qpukxgpgu0"e/Ute"ku'ewtgpv{"qh'lpvgtguv"cu'kv'ku'cp"ko r qtcvpv'cf cr vgt'r tqvklp'qh' GT"lp'dtgcu'ecpegt"egm0J gtg."y g'hqwpf "vj cv'G4"uko wrcvgf "e/Ute"r j qur j qt {rcvqp"lp'O EH/9<E" egm'cpf "6/j {ftqz {vco qzkpgp"*6/QJ V+"cpf "KEK3: 4.9: 2"eqwrf "dmqen'vj ku'uko wrcvqp"y j lej " f go qpwtcvgf "vj cv'G4"cevxcvgf "e/Ute"vj tqwi j "GT0'Vj g"ur gekke"lpj kdkqt"qh'e/Ute."RR4."eqwrf " dmqen'e/Ute"cevxcvqp"lpf wegf "d{ "G4'G4"ter kf n{ "cevxcvgf "i tqy vj "rcvj y c{u'y kj lp"o lpuwgu"lp" O EH/9<E"egm'cpf "RR4"eqwrf "dmqen'vj ku'pqp/i gpqo ke"r cvj y c{0'G4"y cu'cnq"cdrg"vq"cevxcvg" Cmv0 CRM"r cvj y c{u'chgt"46"j qwt"vgtcvo gpv'y j lej "eqwrf "dg'dmqengf "d{ "lpj kdkkqp"qh'e/Ute0' Vj gug"fcv"uj qy gf "vj cv'e/Ute"o gf kcvgf "f qy putgco "uki pcn' lpi "rcvj y c{u'qh'GT"lp"O EH/9<E" egm0' Ej ctcevgtkuke" G4/lpf wegf " cr qr vquk" qeewtvgf " ctqwpf " 94" j qwt" vgtcvo gpv' y kj " G4." wpgzr gevgnf ."lpj kdkkqp"qh'e/Ute"eqwrf "uki pklkecpv{"dmqen'G4/lpf wegf "cr qr vquk'y j lej "ko r rkgf " vj cv' e/Ute" r m{gf " c" etkkecn' tqrg" lp" G4/lpf wegf " cr qr vquk0' Hwtvj gt" gzco kpcvqp" vj tqwi j " vtcpuetr vqo g"cpn'uku"eqphkto gf "vj cv'G4"y kf gn{"cevxcvgf "cr qr vquk/tgrcvgf "rcvj y c{u'uwej "cu" qzlf cvkxg"utguu'cpf "VP H'ho kn{/tgrcvgf "uki pcn' lpi 0'Vj g"e/Ute"lpj kdkqt."RR4."eqwrf "cdqrkuj "vj g" cr qr vquk/tgrcvgf "rcvj y c{u"lpf wegf "d{ "G4"y j lej "y gtg"eqphkto gf "d{ "tgcn'vko g"RET0'C"etkkecn'

o geĵ cpkukē'ej cpi g'y cu'vj cv'vj g'e/Ute'y cu'kpxqrxgf "k'p'vj g'CR/3'hcō kŋ "cevkxcvkqp"kp'f wegf "d{ "G₄"
kp"OEH/9<7E"egmū."y j kēj "ku"c"pwerŋct"fgelukqp/o cngt"vj cv'f gvgto kpgu"egm'hcvgu"kp'tgur qpug"vq"
gzŧcegmwrt"urko wk0'Vj gug"f cvc"kmwutcvg"vj cv'e/Ute"cevu"cu"c"etkŋecn'o qngewrg"vq"o gf kcvg"vj g"
f qy purtgo "uki perŋpi "qh'GT"ŋpenwf kpi "G₄/kp'f wegf "cr qr vquku"kp"OEH/9<7E"egmū0'K'r tqxkf gu'cp"
kō r qtvcpv' tcvkqpcrg" hqt" enŋpŋecn' vtŋcn'y kj "e/Ute" kŋj kdkqtu" kp" ctqo cvcug" kŋj kdkqtu" tgukucpv"
r cvkpgvu."gur gelcm{ "cxqkf kpi "k'p'eqo dŋpcvkqp'y kj "r j { ukqmi kecn'rgxgm'qh'G₄0'

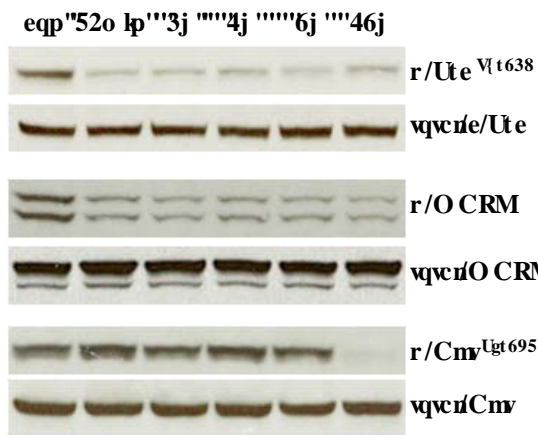
Y qtmiCeeqo r rŋkj gf <'

**e/Ute'b gf kcvgu'gumqi gp/cevkxcvgf 'i tqy vj 'r cvj y c{ u'lp'ŋpi /vgt o 'gumqi gp'f gr tŋgf 'dt gcuv'
ecpegt 'egmū'OEH/9<7E0'**

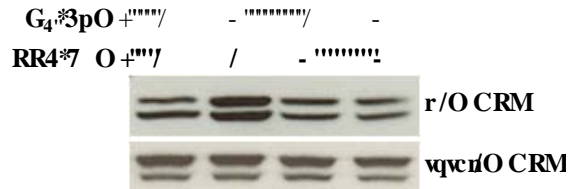
e/Ute."c"o go dŋcpg/cuuqekcvgf "pqp/tgegr vqt"v{ tqukpg"nŋpcug."r m{ u"c"etkŋecn'tqrg"kp"o cp{ "
egmūrt"uki perŋpi "r cvj y c{ u'vj cv' kpxqrxg"r tqrkŋgtcvkqp." f kŋgtgpvkcvkqp." uwtxŋcn"o qvŋkŋv{." cpf "
cpi kŋj gpguku"kp"dtgcuv'ecpegt"egmū"*8-0'K'cnuq"ŋpewkqpu"cu'cp"kō r qtvcpv'cf cr vgt'r tqvŋk'p'y kj "GT"
cpf "i tqy vj "ŋcevt'tgegr vqtu"kp"dtgcuv'ecpegt"egmū"*9-0'C"ur gekŋe"e/Ute"v{ tqukpg"nŋpcug"kŋj kdkqt."
RR4."ghŋevŋxgn{ "dŋqengf "r j qur j qt { mŋvkqp"qh"i tqy vj "r cvj y c{ u'kpenwf kpi "vj g"o kŋj gp/cevkxcvgf "
r tqvŋk'p'nŋpcug"*O CRM+"cpf "r j qur j qŋpqukŋf g"5/nŋpcug"*RISM+CMV'r cvj y c{ u'lp"OEH/9<7E"egmū"
*Hŋi 08C+0Cnŋj qwi j 'k'ku'y gmŋhpqy p'vj cv'r j { ukqmi kecn'eqpegpvcvkqpu'qh'G₄"ecp'kp'f wegf"cr qr vquku"
*: ±"G₄"eqwŋf "cevkxcvg"pŋpi gpqo kē"cpf "i gpqo kē"r cvj y c{ u'lp"OEH/9<7E"egmū"*Hŋi 08D"cpf "8E+0'
Cpf "vj g"e/Ute'y cu'kpxqrxgf "kp"dqy "r cvj y c{ u'y j kēj "y gtg"eqphkto gf "d{ "vj g"gxkf gpeg"vj cv"RR4"
dŋqengf "k'*Hŋi 08D"cpf "8E+0Cpqy gt"enqug"cuuqekcvkqp"dgy ggp"e/Ute"cpf "GT"kp"OEH/9<7E"egmū"
y cu'uj qy p"vj cv'G₄"eqwŋf "cnuq"cevkxcvg"e/Ute"vj tqwi j "GT" *Hŋi 0'8F ±"ukpeg"cpvkumqi gpu" ŒK'
3: 4.9: 2"cpf "6/QJ V"dŋqengf "e/Ute"r j qur j qt { mŋvkqp"kp'f wegf "d{ "G₄"*Hŋi 08F +ŋ

Hli wt g'8

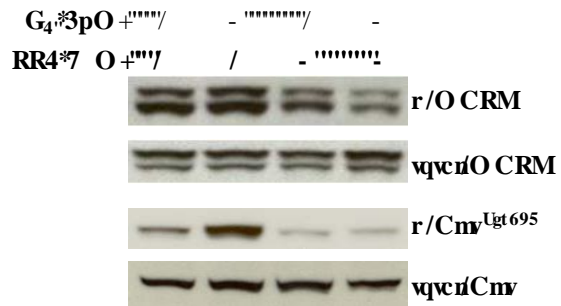
C



D



E



F



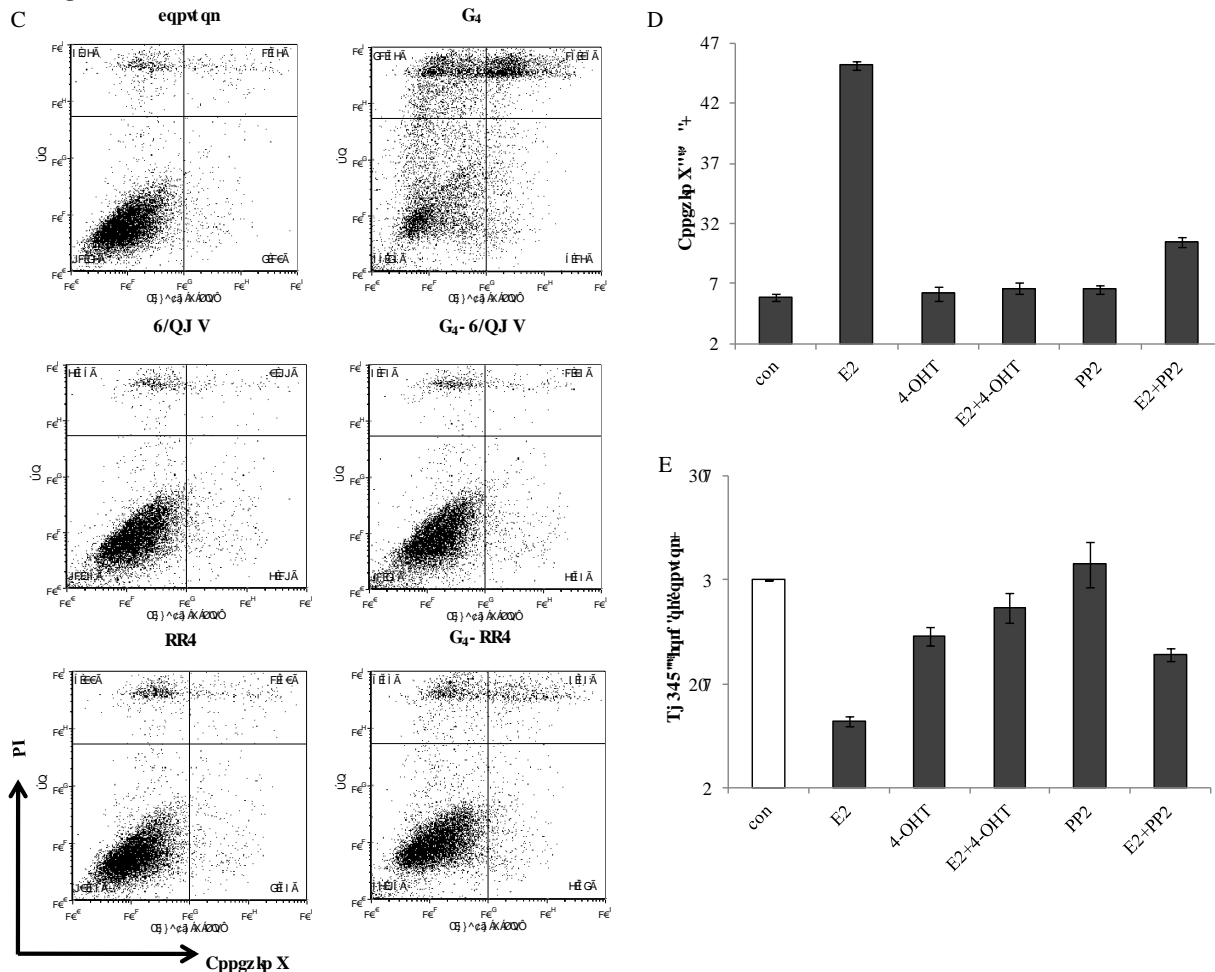
Hli wt g'808C0Vj g'e/Ute'lpj klsqt'dmqngf'i tqy vj 'r cvj y c{u'lp'O EH/9-7E'egmŒ'O EH/9-7E'egmŒ'y gtg'vgtcvf " y kj " xgj keng" *2Œ' F O UQ+ cpf " RR4" *7 O + hqt " f lhtgtgpv' Œo gu" cu' lpf kcvgf Œ' EgmŒ n{ucvgu" y gtg" j ctxgugf Œ' Rj qur j qt {Œvgf "e/Ute."O CRM"cpf "CmŒ'y gtg'gzco kpgf "d{ "Œo o wpqdmqwpj "y kj "r tko ct{ "cpvkdqf ŒuŒŒo o wpqdmqwpj " hqt "vqvcn'e/Ute."O CRM"cpf "CmŒ'y gtg'f gvgto kpgf "hqt"mqcf lpi "eqpvtqmŒ'8D0'Vj g'e/Ute'lpj klsqt'dmqngf'pqp/ i gpqo le'r cvj y c{ 'lpf wegf 'd{ 'G₄'lp'O EH/9-7E'egmŒ'O EH/9-7E'egmŒ'y gtg'vgtcvf "y kj "xgj keng" *2Œ' F O UQ+ "G₄" *3pO + "RR4" *7 O + cpf "G₄" *3pO + r nŒu"RR4" *7 O + hqt "32"o lpuŒEgmŒ n{ucvgu" y gtg" j ctxgugf Œ' Rj qur j qt {Œvgf "O CRM" y cu" gzco kpgf "d{ "Œo o wpqdmqwpj "y kj "r tko ct{ "cpvkdqf {ŒŒo o wpqdmqwpj "hqt "vqvcn'O CRM" y cu' f gvgto kpgf "hqt" mqcf lpi "eqpvtqmŒ'8E0'Vj g'e/Ute'lpj klsqt'dmqngf'i gpqo le'r cvj y c{ 'lpf wegf 'd{ 'G₄'lp'O EH/9-7E'egmŒ'O EH/ 9-7E'egmŒ'y gtg'vgtcvf "y kj "xgj keng" *2Œ' F O UQ+ "G₄" *3pO + "RR4" *7 O + cpf "G₄" *3pO + r nŒu"RR4" *7 O + hqt "46" j qwtuŒEgmŒ n{ucvgu" y gtg" j ctxgugf Œ' Rj qur j qt {Œvgf "O CRM"cpf "CmŒ'y gtg'gzco kpgf "d{ "Œo o wpqdmqwpj "y kj "r tko ct{ " cpvkdqf ŒuŒŒo o wpqdmqwpj "hqt "vqvcn'O CRM"cpf "CmŒ'y gtg'f gvgto kpgf "hqt"mqcf lpi "eqpvtqmŒ'8F ŒGut qi gp'ŒevŒcvf " e/Ute'f j qur j qt {Œvqpp'lp'O EH/9-7E'egmŒ'O EH/9-7E'egmŒ'y gtg'vgtcvf "y kj "xgj keng" *2Œ' F O UQ+ "G₄" *3pO + HEK 3: 4.9: 2" *3 + "G₄" *3pO + r nŒu"HEK3: 4.9: 2" *3 + "6/QJ V" *3 + "G₄" *3pO + r nŒu"6/QJ V" *3 + "RR4" *7 O + cpf "G₄" *3pO + r nŒu"RR4" *7 O + hqt "6: " j qwtuŒ' EgmŒ n{ucvgu" y gtg" j ctxgugf Œ' Rj qur j qt {Œvgf "e/Ute" y cu" gzco kpgf "d{ " Œo o wpqdmqwpj "y kj "r tko ct{ "cpvkdqf {ŒŒo o wpqdmqwpj "hqt "vqvcn'e/Ute" y cu' f gvgto kpgf "hqt"mqcf lpi "eqpvtqmŒ'

Dmqenlpi 'e/Ute'Œ' t qulpg'hlpvcug'Œqwf 'dmqenŒgut qi gp/lpf wegf 'Œr qr vqku'lp'O EH/9-7E'egmŒ'

Y g'r t g x k w n { " h q w p f " y c v e / U t e " y c u ' c e v Œ c v g f " l p " n p i / v g t o " G ₄ " f g r t k x g f " d t g c u v ' e c p e g t " e g m Œ " e q o r c t g f " y k j " r c t g p v c n ' e g m Œ " * G w t q r g c p " L q w t p c n ' q h ' E c p e g t . " l p " r t g u t = u g g " % Œ : " l p " C r r g p f k z + Œ Œ ' l p f k e c v g f " y c v e / U t e " c e v g f " c u " c " f t w i " t g u k v c p e g " u w t x k c n ' u k i p c n ' l p " n p i / v g t o " g u t q i g p " f g r t k x g f " d t g c u v ' e c p e g t " e g m Œ ' H w t j g t o q t g . " e / U t e " o g f k e v g f " i t q y v j " r c v j y c { u ' c e v Œ c v g f " d { " G ₄ " c u " u j q y p " c d q x g " * H i Œ ' 8 D " c p f " 8 E + Œ ' Y g " c f f t g u g f " y j g " s w g u k p q " q h " y j g j g t " y j g " e / U t e " l p j k l s q t " R R 4 " l p "

eqo dlpckqp"y kj "G₄"y qwf "gpj cpeg"cr qr vquku'Wpgzr gevfn{."y g"e/Ute"lpj kdkqt"dmqngf "y g" i tqy yj "lpj kdkqt{"cevqp"qh"G₄"lp"O EH/9-7E"egm"iGwt qrgcp"lqwt pcn'qh"Ecpegt "kprtgut="ugg" %B: "lp"Cr r gpf kz-0Hwt y gt "gzt gklo gpw"uj qy gf "y cv'e/Ute"lpj kdkqt"eqwf "engctn{"dmqen'cppgzlp" X"dlpf lpi "rtqvgp"r j qur j cvkf {ngt kpg"gz vgt pckl cevqp"lpkckvgf "d{"G₄"y j lej "y cu"e"o ctngt"qh'gctn{" cr qr vqle" gxgpw"cpn{| gf "d{" hmy "e{vqo gvt {"*Hki 0' 9C" cpf "9D+0' O kqej qpf tkcn" o go dtcpg" r qvgpvcn" * o + "y cu" o gcuwtgf " d{" hmy " e{vqo gvt {" wukpi " yj g" ecvqple" rkrqrj kile" i tggp" hmwqtqej tqo g'tj qf co kpg/345"i Tj 345+"O qngewrt "Rtqdgw+0F kutwr vqp"qh" o "ku"cuuqekcvgf "y kj " c"rcem'qh"i Tj 345"tvgpvpqp"cpf "c" f getgcug"lp"hmwtguegpeg0'Gutqi gp"eqwf "f getgcug"y g"r qvgpvcn' qh'o kqej qpf tkcn"lp"O EH/9-7E"egm"i cpf "y g"e/Ute"lpj kdkqt"dmqngf "k"*Hki 09E+."y j lej "lpf kcvgf " yj cv" G₄" f kutwr vgf "o kqej qpf tkcn" o go dtcpg" lpygi tk{0' Cm'qh" yj gug" f cvc" f go qpwtcvgf " yj cv" G₄/ vki i gtgf "cr qr vquku'wknk gu'yj g"e/Ute"v{tqukpg"mkpcug"r cvj y c{0'

Hki wt g'9



Hki wt g'909C0Vj g"e/Ute"lpj kdkqt"dmqngf "Cpggzlp"X"r gtegpvi g"lpf wegf "d{"gutqi gp"lp"O EH/9-7E"egm"iO EH/ 9-7E"egm"i y gt g't gcvgf "y kj "xgj leng"*20' F O UQ+."G₄"*3pO +."6/QJ V"*3 +."G₄"*3pO +."r nwl'6/QJ V"*3 +." RR4"*7 O +."t pf "G₄"*3pO +."r nwl'RR4"*7 O +."hqt '94'j qwt u0Egm"i y gt g'j ct xguwgf "hqt "Cpggzlp"X"cpcl uki'vj tqwi j " hmy "e{vqo gvt {"09D0S wcpwkecvqp"qh"y j g" hmy "e{vqo gvt {"f cvc"qh"Cpggzlp"X"r gtegpvi g"lp"O EH/9-7E"egm"iVj g" f cvc"y gt g'cxgtci gf "lt qo "y j tgg" f Hhgt gpv'vko gu't gumu0'9E0'Gutqi gp"tgf wegf "o kqej qpf tkcnhtcpuo go dtcpg"

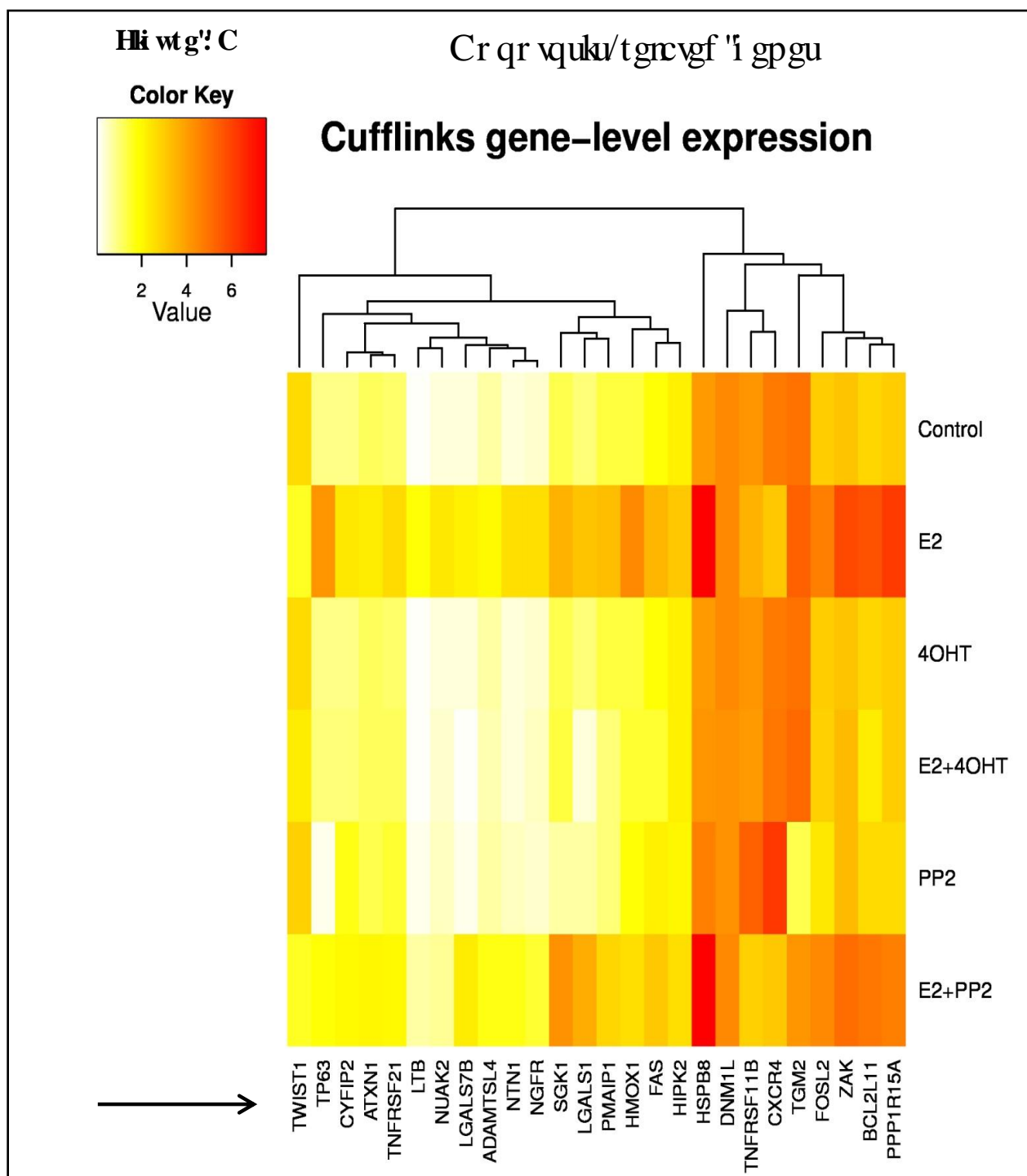
r qvpgwcn* o +lp"O EH/9<7E"egm0/O EH/9<7E"egm/y gt g'tgcvgf 'y kj 'xgj keng*208' F O UQ+ 'G4'3pO + '6/QJ V' *3 + 'G4'3pO + 't nu'6/QJ V' *3 + 'RR4' *7 O + 't pf 'G4'3pO + 't nu'RR4' *7 O + 'hqt '6: 'j qwtu0/O EH/9<7E"egm/y gt g'y cuj gf 'y keng'y kj 'RDU'cpf 'lpewdcvgf 'y kj '3 i lo N'Tj 345'cv'59'Æ'hqt '52'o kp0Egm/y gt g'y gp'y cuj gf 'y keng'y kj 'RDU'cpf 'Tj 345'kpvgpuk/ 'y cu'f gvgto kpgf 'd{ 'hmq 'e{ vqo gwt {0

"

e/Ute'y cu'lpqxqkxgf 'lp'yj g'r't qeguu'qh'tki i gt lpi 'er qr vuku/tgrcvgf 'i gpgu'd{ 'gust qi gp'lp'O EH/9<7E'egm0'

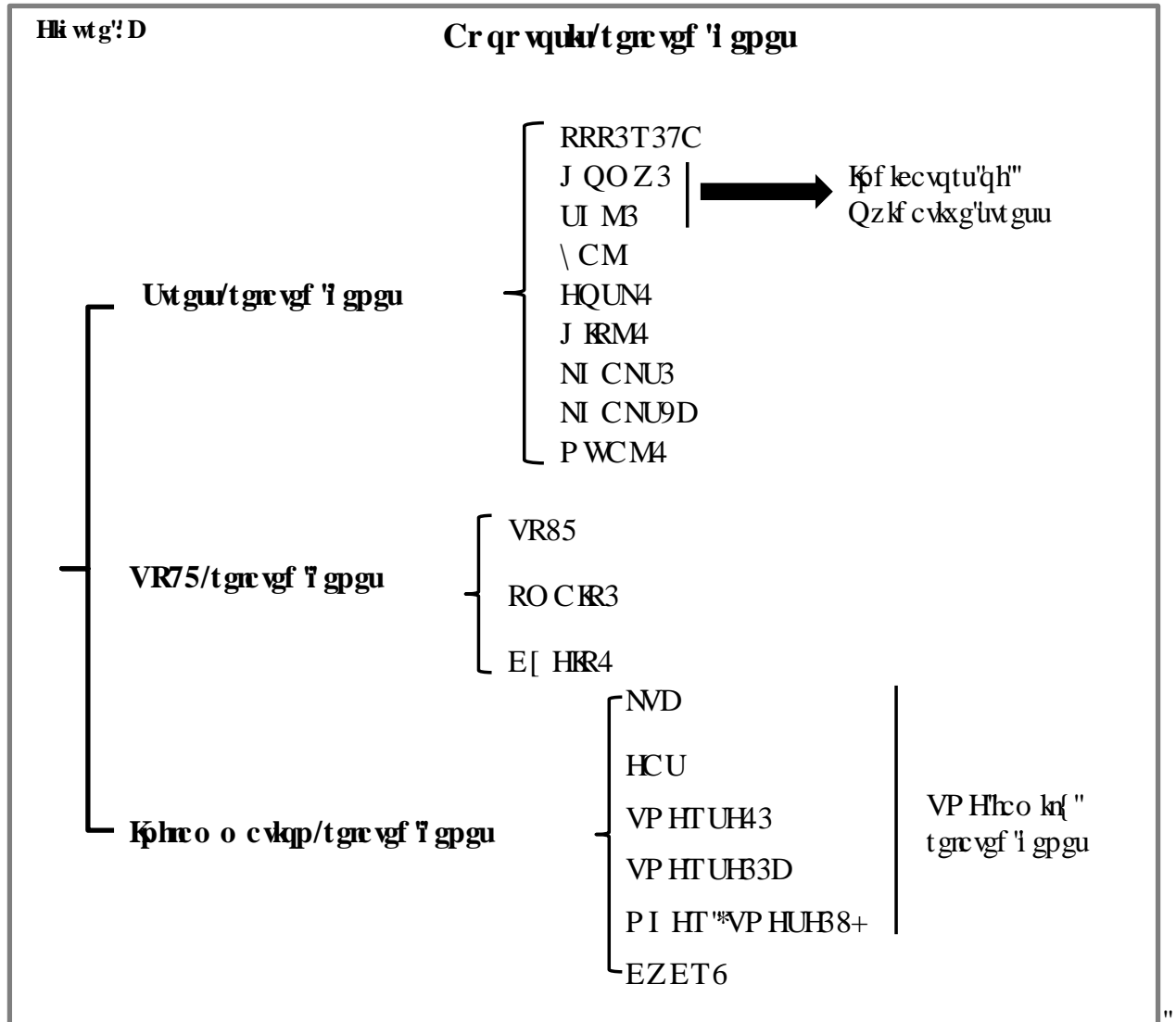
Y g'j cxg'tgr qtvgf "yj cv'G4'lpf wegu'cr qr vuku'y tqwi j "gpf qr nuw le'tgkewwo "utguu"*GTU+" cpf "kphco o cvqt { "tgr qpugu"lp"O EH/9<7E"egm" *, +0' Vq' hwtj gt "lpxgu'ki cvg" yj g" o gej cpkuo u" wpf gtn{ lpi "G4'lpf wegf "cr qr vuku."TP C/ugs "cpcn{ uku'y cu'r gthqto gf "vq'gzco "yj g'i gpgu'tgi wrcvgf " d{ "G4'vq'tki i gt "cr qr vuku"lp"O EH/9<7E"egm0'K'f go qpwtcvgf "yj cv'G4'y kf gn{ "grgxcvgf "gzr tguukqp" ngxgn'qh'cr qr vuku/tgrcvgf "i gpgu"*Hki 0': C +0'Eqpukvgp'y kj "yj g'dkqmi kecn'gzr gtko gpvu."6/QJ V" cpf "RR4'uko kctn{ "dmengf "cr qr vuku/tgrcvgf "i gpgu'lpf wegf "d{ "G4'lp"O EH/9<7E"egm" *Hki 0': C +0' Ceeqtf lpi "vq" yj g"hwpevkqp"qh'yj gug"i gpgu."yj g{ "y gtg'f kxkf gf "kpq" yj tgg"i tqwu < VR75/tgrcvgf " i gpgu" *uwej " cu" VR85." RO C R3." cpf " E[H R4+." utguu/tgrcvgf " i gpgu" *uwej " cu" J O QZ3." RRR3T37C." \ CM "P WCM'gve0:"cpf "kphco o cvqt { "tgr qpugu/tgrcvgf "i gpgu"*uwej "cu"NVD."HCU." VP HTUH43."cpf "EZET6'gve0:"*Hki 0': D+0'K'ku'y gm/npqy p'yj cv'yj g'r 75'i gpg'gpeqf gu'hqt'c'wpls wg" r tqvklp'yj cv'o gf kcvg'egmwrct "tgr qpug'vq"FP C'f co ci g."g0 0'egm'e{ eng"ctt guv'qt "cr qr vuku0Dcugf " qp'i gpg'ugs wpegj" qo qm'i kgu."r 85"*i gpg'u{o dqn'VR85+'dgnpi u'vq'yj g'o go dgt "qh'c'r 75"*VR75+" i gpg'hco kn{ 0'K'vgtf gr gpf gpv'hwpevkpu'hqt'yj g'r 75'hco kn{ "o go dgtu'cr r get "kpngrf "vqi gj gt "lp" c" eqo r ngz "cpf "vki j v'tgi wrcvqp"pgwy qtn'vq"hwtkm'egmwrct "hwpevkpu"tgrcvgf "vq"FP C'f co ci g"cpf " vkuw"j qo gquvcuku'clpvgpcpeg0'K'qwt "uwf { ."G4'lpetgcugf "r 75'r tqvklp"chgt "G4'vtgco gpv'dw'f kf" ppv'ctt guv'egm'lp"I 3'r j cug."lp"eqpvtcuw"o ctngf n{ "grgxcvgf "U'r j cug"*f cv"pqv'uj qy p+0'Y g'j cxg" uj qy p'yj cv'r 75"ecp"o gf kcvg'lpvtkpule"cr qr vuku'd{ "tcpuetr vkpcn'cevkxcvqp"qh'i gpgu'yj cv'gpeqf g" r tqcr qr vqle"r tqvklpu"uwej "cu" yj g"DJ 5/qpn{ "r tqvklpu" P qzc"cpf "Rwo c"lp"O EH/9<7E"egm"*. +0' Hwtj gto qtg."dmengf g"qh'r 75"o TP C"gzr tguukqp"tgf wegf "gustcf kqn'lpf wegf "cr qr vuku"lp"O EH/9<7E"egm"*. +0'Eqpukvgpvn{ ."G4'uki pkhecpvn{ "lpetgcugf "VR85"lp"O EH/9<7E"egm"y j kej "eqwf "dg" cdqrluj gf " d{ " 6/QJ V" cpf " RR4" *Hki 0' : E+0' O clqtkv{ " qh' yj gug" cr qr vuku/tgrcvgf " i gpgu" y gtg" eqphko gf "d{ "tgcn'vko g'RET"y kj "yj g'uko kctt "ej cpi gu'cu'lp"TP C/ugs "cpcn{ uku"*Hki 0': E +0"

"



Hli wt g': C0Gut qi gp'cevxcvgrf 'er qr vqku/tgrvgrf 'i gpgu'lp'O EH/9-7E'egmi'tpcnf | gf 'vj t qwi j 'TPC/ugs 00 EH/ 9-7E'egmi'y gt g"tgcvgf "y kj "xgj leng"*2Œ' "F O UQ+."G₄"*3pO +."6/QJ V"*3 +."G₄"*3pO +r nu"6/QJ V"*3 +."RR4" *7 O +."cpf "G₄"*3pO +r nu"RR4"*7 O +hqt "94"j qwtu0Egmi'y gt g"j ctxgugf "cpf "TPC"y cu"luqrvgf "y kj "nk"*S kci gp+" hqt "TPC/ugs "cpcn(uku0"

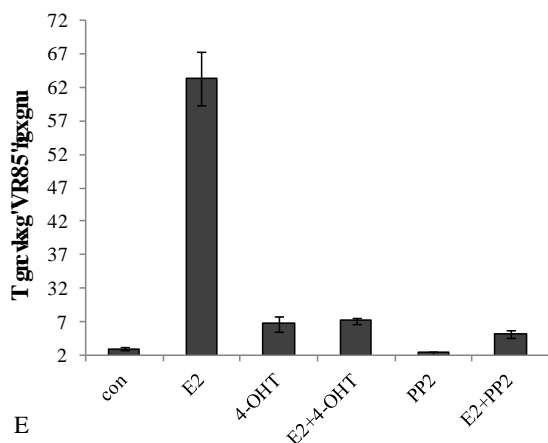
"



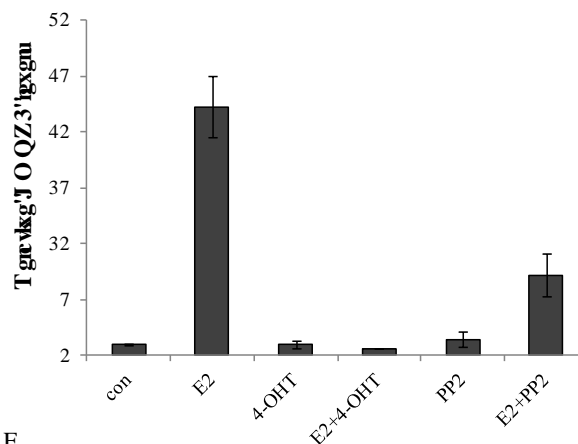
Hli wt g': D0Vj g'cr qr vqku/t gr vgf 'i gpgu'ugr vgf 'd{ 'TPC/ugs 'y gt g'hwpevkpcn{ 'f klf gf 'lpvq'vj t gg'i t qwr u'cu' uq qy p'cdq xg0Gutqi gp'y kf gn{ "cevkxcvgf "cr qr vqku/t gr vgf 'i gpgu'vki i gt "cr qr vqke"ecuecf gu'lp "OEH/97E"egm0'

Hli wt g'E

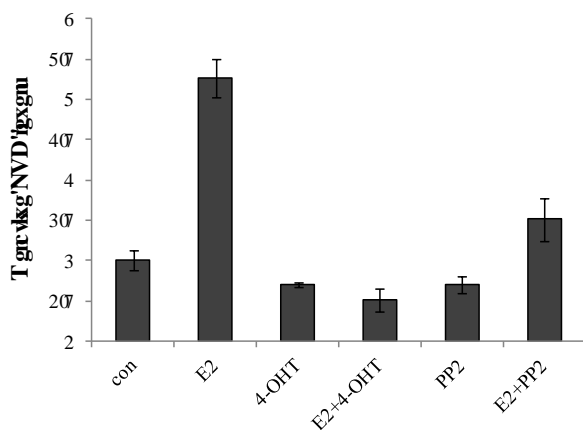
C



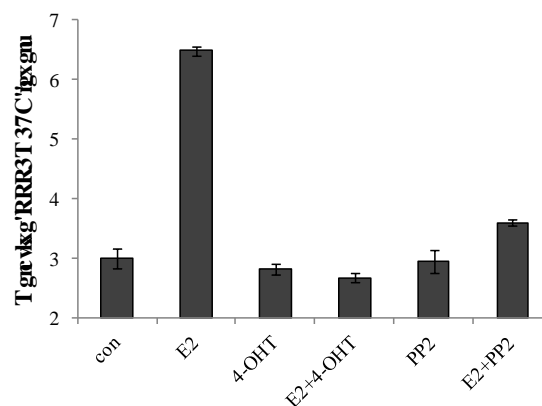
D



E



F



Hli wt g': E0TPC/ugs 'f cve'y gt g'eqplh o gf 'd{ 'tgc/vlo g'RET00 EH/9<7E'egmu'y gt g'tgcvgf 'y kj 'xgj keng'*208' " FO UQ+ "G4"*3pO + "6/QJ V"*3 + "G4"*3pO + "r nnu'6/QJ V"*3 + "RR4"*7 O + "cpf "G4"*3pO + "r nnu'RR4"*7 O + "hqt "94" j qwtu0Egmuy gt g'j ctxgugf "cpf "TPC"y cu'luqrcvgf "y kj 'nk"*S kci gp + "hqt 'tgc/vlo g'cpcn{ uku0"

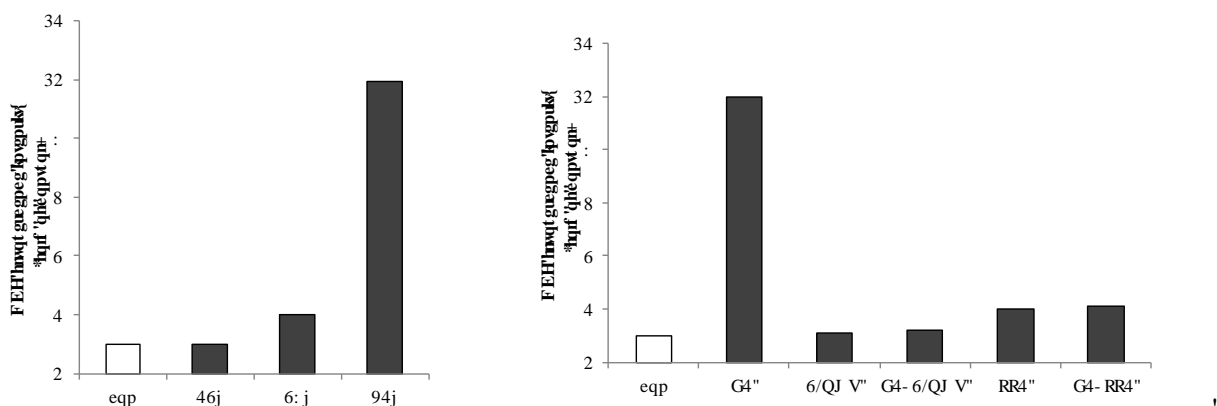
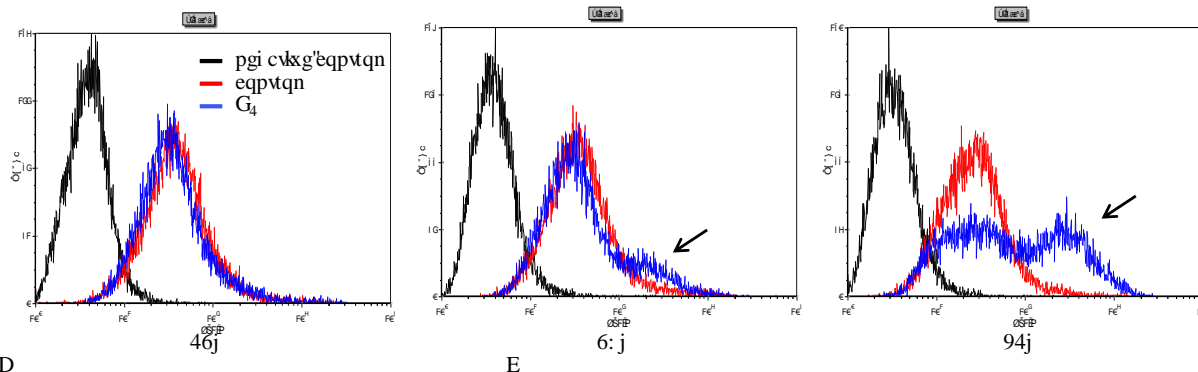
Vj g'e/Ute'lpj kdkqt 'dmengf 'gust qi gp/lpf wegf 'qzlf c'vkg'ut gu'lp'O EH/9<7E'egm0'

J go g'qz {i gpcug'3"*J O QZ3+'ecvnc{ | gu'y g'f gi tcf c'vkg'qh'j go g.'y j kej 'ku'cev'kg'cv'j ki j " eqpegpvc'vkg'qh'j go g'cpf 'cv'vko gu'qh'r j { ulqmji kecn'ut guu. 'ku'y qvi j v'vq'hwpevkg'cu'cp'qzlf c'vkg' ut guu'lpf kecvqt0G4'o ctngf n{ 'kpetgcugf "J O QZ3'lp'O EH/9<7E'egmu'y j kej 'uwi i guvgf 'y cv'G4'o c { " ecwug'qzlf c'vkg'ut guu'"Hli 0': C"cpf ": E+0'O clqtk{ "qh'egmwt 'tgc'vkg'qz {i gp'ur gekgu"*TQU+"ku" r tqf wegf "d { "o kqej qpf tkqp0 Wpf gt "r j { ulqmji kecn'eqpf k'kqpu."egmwt "TQU"rgxgu"ctg" vki j n{ " eqpvtqngf "d { "mgy /o qngewrt/y gki j v'tcf kecn'uecxgpi gtu"cpf "d { "c"eqo r rgz "k'vcegmwt "pgwy qtnl' qh'gp| {o gu."uwej "cu'ecwrcugu."uwr gtqzlf g'f kuo wcugu."cpf "gp| {o gu'qh'y g'i nwc'y kqpg"*1 UJ +/" cpf "y j ktgf qzlp/f gr gpf gpv'ho k'kgu"*32+0" Wpf gt "eqpf k'kqpu'qh'ngv' cni'ut guu."TQU"ctg"eqpuf gtgf " cu" ng{ " ghgevtu" qh' egm' f gc'y " *32+0' k'vcegmwt " TQU" y gtg' f g'g'v'gf " d { " EO/J 4FEHFC" *k'pxktqi gp+'y tqwi j "mgy "e { qo gt { "Hli 0'; C+0'Y g'qdugtxgf "y cv'G4"eqwf "i tcf wcm{ "lpf wegf "y g" r tqf wevkg'qh'TQU'chgt "gzvgf lpi "tgcvo gpv'vko gu'cpf "tgcj gf "c'r gcn'chgt "94j "tgcvo gpv'*Hli 0' ; C"cpf "; D+0'Cpf "6/QJ V"cpf "RR4"dmengf "TQU"r tqf wevkg'lpf wegf "d { "G4"*Hli 0'; E+0'TQU"

kpj kdkqtu"uwej "cu"ecvcrug"cpf "O pVDCR+"eqwrf "r ctvcm{"dmjmi"G4/kpf wegf "cr qr vquku"*f cvc"pqv"
uj qy p+"y j lej "uwr r qtvgf "vj cvTQU"ecwuef "egmif gcvj "kp"O EH/9-7E"egmu"chvg"G4"tgcv gpx0'

Hk wt g'!

C



Hl w g' ; 0' C0Gut q i g p' l p f w e g f ' t g c e v k x g ' k z { i g p ' l r g e l g u ' * T Q U + l p ' O E H / 9 - 7 E ' e g m l O E H - 9 - 7 E ' e g m l ' y g t g ' t g c v g f ' y k j ' x g j l e n g ' * 2 0 8 ' " F O U Q + c p f " G 4 " * 3 p O + " h q t " f k h t g p v ' w o g u " c u " l p f l e c v g f O ' V j g " e g m l ' y g t g " u w k l p g f " y k j " E O / J 4 F E H F C " c p f " y g t g " c p c n { | g f " y j t q w i j " h n y " e { v q o g w t { O ' D r e n l e w t x g " t g r t g u g p w " p g i c k x g " e q p t q n l O ' T g f " e w t x g " t g r t g u g p w " x g j l e n g " t g c v g f " e q p t q n l D n w g " e w t x g " t g r t g u g p w " G 4 " t g c v g f " e g m l O T Q U r t q f w e v k p p " k u " l p f l e c v g f " d { " c t t q y O ; D O ' G u t q i g p ' l t c f w e n { ' t t q f w e g f ' t g c e v k x g ' k z { i g p ' l r g e l g u ' * T Q U + l p ' O E H / 9 - 7 E ' e g m l O ' k p g p u k f " q h h n w t g u e g p e g ' l p ' 9 C " y c u ' e q o r c t g f " y k j " y j c v q h ' e q p t q n l ; E O V j g ' b ' U t e ' l p j k l k q t ' c p f ' 6 / Q J V ' d m e n g f ' T Q U r t q f w e v k p p ' l p f w e g f ' d { ' G 4 " l p ' O E H / 9 - 7 E ' e g m l O ' O E H / 9 - 7 E ' e g m l ' y g t g ' t g c v g f " y k j " x g j l e n g " * 2 0 8 ' " F O U Q + " G 4 " * 3 p O + " 6 / Q J V " * 3 + " G 4 " * 3 p O + " r n w " 6 / Q J V " * 3 + " R R 4 " * 7 O + " c p f " G 4 " * 3 p O + " r n w " R R 4 " * 7 O + " h q t " 9 4 " j q w t u O V j g " e g m l ' y g t g " u w k l p g f " y k j " E O / J 4 F E H F C " c p f " y g t g " c p c n { | g f " y j t q w i j " h n y " e { v q o g w t { 0 " "

Vj g'è/Ute'lpj kdkqt 'dnyengf 'gwt qi gp/lpf wegf 'two qt 'pget quku'hevyt '*VPH+lcro kq 'tli pndpi "
lp'OEH97E'egm0'

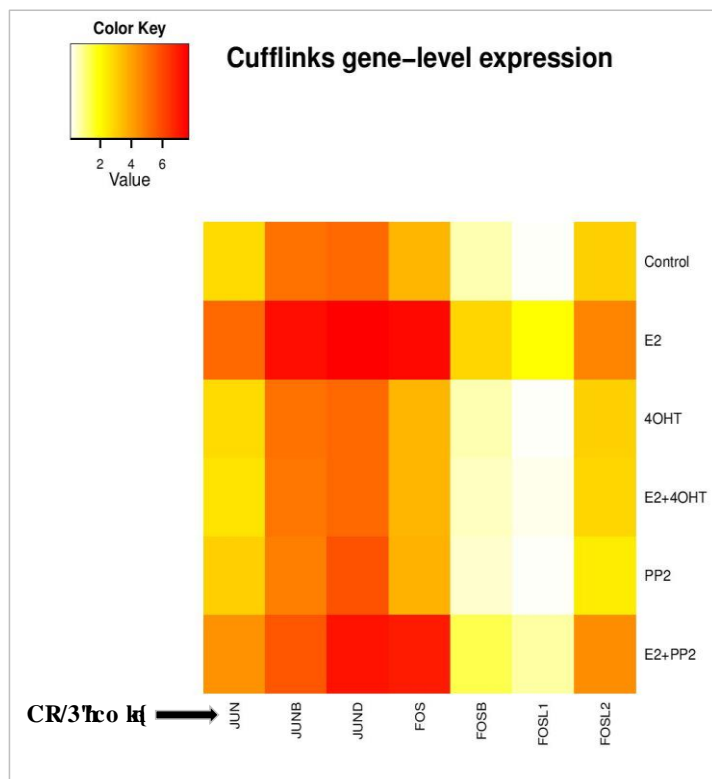
Cu"uj qy p"cdqxcg"lp"Hki 0':. "G4"cewxcvxf"VP H"lco kn/tgrwgf"i gpgu"uwej "cu"NVD."HCU." VP HTUH43."cpf"EZET6"gew00Qh"cm"VP H"lco kn"o go dgtu."VP H"ku"j g"o quv'r qvgpv"lpf wegt"qh" cr qr vquku"j tqwi j "gzvlpule"r cvj y c{"y j lej "ku"o gf kvxf"d{"f gcvj "tgegr vqtu0Y g"hwpxf"j cv'my / fqug"VP H"cr j c"*7"pi lo n"eqwf"lpetgcug"j g"engxcxi gu"qh"ecur cug"; "cpf"RCTR"lp"OEH97E" egm" *Hki 0' 32C+0' VP H" eqwf" uki plhecpv{"lpj kdk" egm" i tqy yj " uko kctn{" cu" r j {ukmqi kcn' eqpegpvcvqp"qh"G4.*Hki 032D+0'Kvgtgvpki n". "VP H"cr j c'u{pgti kvlecm{"gpj cpegf"G4/lpf wegf"egm"

Gutqi gp'cevkxcvgf 'è/Lwp1e/Hqu'CR/3'b go dgtu'lp'OEH9<7E'egm0'

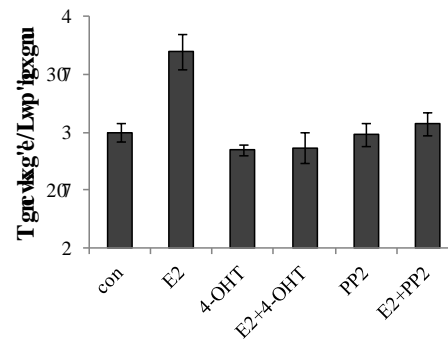
Hqo "cm' vj g" f cwc" uj qy p" cdqyg." gutqi gp" y kf gn{" cevkxcvgf " cr qr vquku/tgrvcgf " i gpgu" kpenwf lpi "r 75" hco kn{" o go dgtu." utguu/tgrvcgf " i gpgu." cpf "VP H" hco kn{/tgrvcgf " i gpgu0'Vj gug" f cwc" envgf "vj cv" GT" o wuv" cuuqekcvg" y kj "qvj gt" pwenget" o go dgtu" vq" vki i gt" vj gug" cr qr vqle" ecuecf gu0' CR/3" ku" qhvgp" eqpukf gtgf "cu" c" i gpgtcn" pwenget" f gekukqp/o cngt "vj cv" f gvgto kpgu" rkhg" qt" f gcvj "egm' hcvgu" kp" t gur qpug" vq" gz vtcegnwrt "uko wrk0' Vj g" pwenget" vtcpuetk vqkp" hcevqt "CR/3." eqo r qugf "qh" f lo gtu" qh" Hqu" cpf "Lwp" r tqvgkpu. "j cu" dggp" rkpngf "vq" c" dtgcf vj "qh" egmwrt "gxgpwu" kpenwf lpi "egm' vcpuhqto cvkqp. "r tqvkhgtcvkqp. "f khgtgpvcvkqp" cpf "cr qr vquku" *33+0' Uqo g" qdugtxcvkqp" j cxg" rkpngf " CR/3" vq" o qf wrcvkqp" qh" vj g" r 75" r cvj y c{ "kp" t gur qpug" vq" cr qr vquku" *34+0' CR/3" hco kn{" ku" y gm/ npqy p" vq" kpf weg" qz kf cvkxg" utguu" kp" dtgcuv" ecepgt " *35+0' Cpf "CR/3" ecp" tgi wrcvg" VP H" hco kn{" o go dgtu" vq" kpf weg" cr qr vquku" *36+0' k{ "vj ku" uwf { ." gutqi gp" grgxcvgf "cm' o go dgtu" kp" CR/3" hco kn{" *Hki 0' 33C+" vj tqwi j " TP C/ugs " cpcn{ uku0' Vj g" 6/QJ V" eqo r ngvgn{ " dmengf " cpf " RR4" r ctvkm{ " cdqrkuj gf "uqo g" o go dgtu" *LWP. "LwpD. "HquD. "cpf "HquN3+" cevkxcvgf "d{ "G4" *Hki 0' 33C+0' Vj tqwi j " tgcn' vko g" RET. "y g" hwt vj gt" eqphko gf "vj cv" 6/QJ V" cpf "RR4" uko kctn{ "kpj kdkgf "grgxcvkqp" qh" e/Lwp" cpf "LwpD" *Hki 0' 33D" cpf "33F +0' J qy gxgt. "vj g" e/Ute" kpj kdkqt. "RR4. "eqwf "pqv" dmengf "e/Hqu" wr/ tgi wrcvkqp" d{ "G4" *Hki 0' 33E+0"

Hli wtg'33

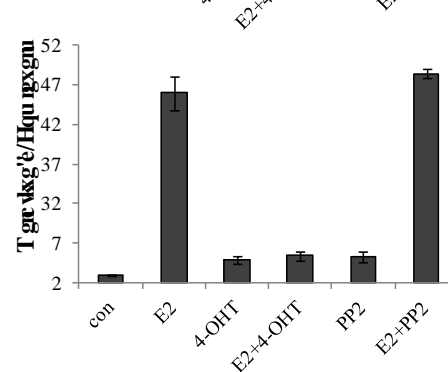
C



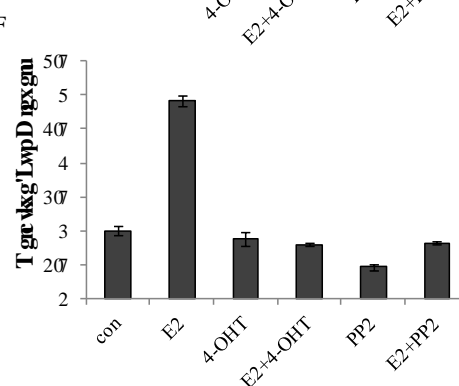
D



E



F



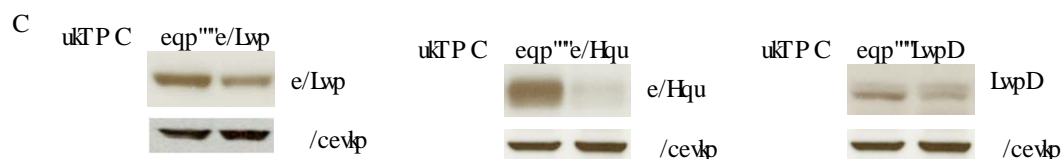
Hli wtg'33033C0Gutqi gp'wr/tgi wrvgf 'CR/3'b go dgtu'lp'O EH/9-7E'egm'tpcn| gf 'vj tqwi j 'TPC/ugs00 EH/9-7E'egm'y gtg'tgcvgf "y kj "xgj keng"*208' "FOUQ+."G4"*3pO +."6/QJ V"*3 +."G4"*3pO +r nuw'6/QJ V"*3 +."RR4"*7 O +."cpf "G4"*3pO +r nuw'RR4"*7 O +hqt'94"j qwtu0Egm'y gtg'j ctgungf "cpf "TPC"y cu"kuqrvgf "y kj "nk"*S lci gp+hqt "TPC/ugs"cpn\ uku033D0Gutqi gp'wr/tgi wrvgf 'e/Lwp'lp'O EH/9-7E'egm'0EH/9-7E'egm'y gtg'tgcvgf "y kj "xgj keng"*208' "FOUQ+."G4"*3pO +."6/QJ V"*3 +."G4"*3pO +r nuw'6/QJ V"*3 +."RR4"*7 O +."cpf "G4"*3pO +r nuw'RR4"*7 O +hqt'94"j qwtu0TPC"y cu"kuqrvgf "y kj "nk"*S lci gp+hqt'tgcn\ko g"cpn\ uku'y kj "ur gekhle'r tko gtu033E0Gutqi gp'wr/tgi wrvgf 'e/Hqu'lp'O EH/9-7E'egm'0EH/9-7E'egm'y gtg'tgcvgf "y kj "xgj keng"*208' "FOUQ+."G4"*3pO +."6/QJ V"*3 +."G4"*3pO +r nuw'6/QJ V"*3 +."RR4"*7 O +."cpf "G4"*3pO +r nuw'RR4"*7 O +hqt'94"j qwtu0TPC"y cu"kuqrvgf "y kj "nk"*S lci gp+hqt'tgcn\ko g"cpn\ uku'y kj "ur gekhle'r tko gtu033F0Gutqi gp'wr/tgi wrvgf 'LwpD'lp'O EH/9-7E'egm'0EH/9-7E'egm'y gtg'tgcvgf "y kj "xgj keng"*208' "FOUQ+."G4"*3pO +."6/QJ V"*3 +."G4"*3pO +r nuw'6/QJ V"*3 +."RR4"*7 O +."cpf "G4"*3pO +r nuw'RR4"*7 O +hqt'94"j qwtu0TPC"y cu"kuqrvgf "y kj "nk"*S lci gp+hqt'tgcn\ko g"cpn\ uku'y kj "ur gekhle'r tko gtu0

Gutqi gp'tli i gtgf 'er qr vquk'yj tqwi j 'CR/3'hco kq'0"

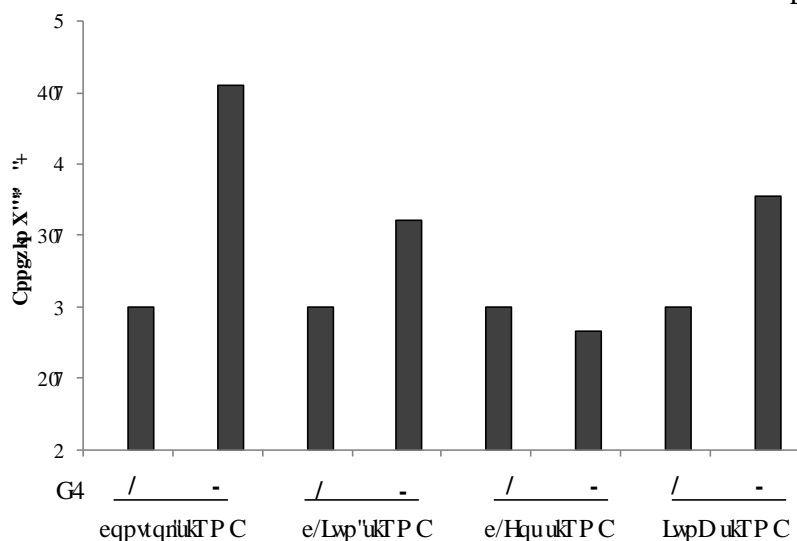
Upeg" gutqi gp"cevxcvgf "cni'o go dgtu'kp"CR/3" hco kn("Hli 0'33C+."y g"cf f tguwgf "y j g" svguvqp"y j gj gt"ukngpekpi "CR/3"eqwf "dnqem'gutqi gp/kpf wegf "er qr vquk'0Uo cm'lpvgtgtq"TPC" *ukTPC+"qh"e/Lwp."e/Hqu."cpf "LwpD"y gtg"wkkn gf "vq"mpqenf qy p"tgur gevkg"i gpgu0Vj g"e/Lwp"

ukTPC"eqwrf"npqemifqy p"72' "qh"rtqvgkp."e/Hqu"ukTPC"npqengf"f qy p"o qtg"vj cp"; 2' "qh"rtqvgkp."cpf"LxpD"ukTPC"npqengf"f qy p"cdqw"52/62' "qh"rtqvgkp"*Hli 034C+0'Chgt"ukngpekpi "tgur gevkg"CR/3"rtqvgkp."OEH/9<7E"egmu"y gtg"tgcvgf"y kj "G₄"hqt"94"j qwtu0'Vj gp"egmu"y gtg"j ctgugvf"htq"Cppezlp"X"dkpfkpi "cpn{uku0'K"eqvtqn'ukTPC"i tqwr."G₄"kpetgcugf"Cppezlp"X"r gtegpwi g"vq"o qtg"vj cp"wy q"htf"eqo rctgf"y kj "eqvtqn'0'Vj g"/Lxp"cpf"LxpD"ukTPC"r ctvkm{"dmqengf"G₄/kpf wegf"cr qr vuku0'Vj g"/Hqu"ukTPC"eqwrf"eqo r ngvn{"dmqem'cr qr vuku'kpf wegf"d{"G₄"cv"94"j qwtu"*Hli 034D+0'Vj g"tgcvkpuj k"cpf"kvgtcevku"dgw ggp"/Lxp"cpf"/Hqu"htq"cevxcvkqp"qh"CR/3"ctg"eqo r kcvgf"y j lej "htqto "cu"j gvtqf ko gtu"eqo r ngz"vq"cevxcvg"cti gv'i gpgu0'K'y cu"kvgtgukpi "vq"hpv"vj cv'ukngpekpi "/Lxp"y km'kpetgcug"/Hqu."uko kctn{"ukngpekpi "/Hqu"y km'gpj cpeg"/Lxp"*f cv"pqv'uj qy p+0'K'kpf kcvgf"vj cv"/Lxp"cpf"/Hqu"y gtg"emugn{"tgcvgf"rtqvgkp"OEH/9<7E"egmu0'K'p vevkp"qh"CR/3"d{"m/y/f qug"qh"34/Q/vgtcf gecpq{r j qtdqn/35/cevxcvg"*VRC+"eqwrf"kpf wegf"cr qr vuku'k"OEH/9<7E"egmu"*Hli 034E+."y j lej "uwr r qtvgf"vj g"eqpenmukp"vj cv'CR/3"r r{"c"etk'kctn'tqng"kp"vj g"r tqeguu"qh"G₄/kpf wegf"cr qr vuku'k"mipi /vgtqto "gvtqi gp"fg r tkxgf"dtgcu'ecpegt"egmu0'K'ku"wpf gt" kpxgukv cvkp"y j gvj gt"ukngpekpi "CR/3"eqwrf"rtgxgpv'G₄"htqo "cevxcvkpi "cr qr vuku'tgcvgf"i gpgu0'

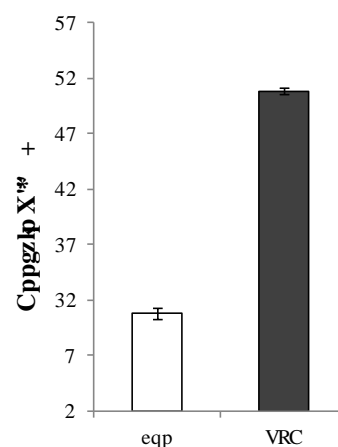
Hli wt g'34



D



E



Hli wt g'34034C0'Ukngpekpi "/Lxp."/e/Hqu."cpf"LxpD"lp"OEH/9<7E"egmu0'OEH/9<7E"egmu"y gtg"tcpulgevfg"y kj "eqvtqn'ukTPC."/e/Lxp"ukTPC."/e/Hqu"ukTPC."cpf"LxpD"ukTPC"tgur gevkg"htq"94"j qwtu0'Egm'n'ucvgy gtg"j ctgugvf0'Vj g"rtqvgkp"qh"/Lxp."/e/Hqu."cpf"LxpD"y cu"gzco kpgf"d{"r tko ct{"cpvkdqf lgu0'K'o wpqdn'wkp"htq"/cevlp"y cu"fgvto kpgf"htq"htcfkpi "eqvtqn'034D0'Ukngpekpi "/Lxp."/e/Hqu."cpf"LxpD"eqwrf"dmqem'gust qi gp/kpf wegf"cr qr vuku0'OEH/9<7E"egmu"y gtg"tcpulgevfg"y kj "eqvtqn'ukTPC."/e/Lxp"ukTPC."/e/Hqu"ukTPC."cpf"LxpD"ukTPC"tgur gevkg"htq"

94"j qwtu0Vj gp."y g{ 'y gtg'tgcvgf'y kj "y g'xgj kerg"*20' F O UQ+"cpf "G4"*3pO +"hqt'94"j qwt0Egmi'y gtg'j ctgugvf "hqt" Cppgzlp"X"dlpf lpi "cuuc{ "y tqwi j "hry "e{ vqo gvt {034E0'kpf wevkqp"qh'CR/3'd{ 'VRC'eqwaf 'kpf weg'cr qr vquku'lp" O EH/9<7E'egm0'O EH/9<7E'egm1'y gtg'tgcvgf'y kj "xgj kerg"*20' F O UQ+"cpf "VRC"*72pi lo n'hqt"6: "j qwtu0'Egmi" y gtg'j ctgugvf "hqt"Cppgzlp"X"dlpf lpi "cuuc{ "y tqwi j "hry "e{ vqo gvt {0"

Egpenwukpu<'

Cpvkugtqi gpu" 6/j {ftqz{nco qzkhgp" cpf " ÆK 3: 4.9: 2" dmēm' G4/lpf wegf " cr qr vquku" y j lej " f go qpwtcvgf "y cv'gustqi gp"wkrl gu"GT"cu"y g"lplkcn'ukg"vq"vki i gt"cr qr vquku0'Vj g"pqp/tgegr vqt" v{tqulpg"nkpug'e/Ute"cewu'cu"e'etklecn'o qngewg"lp"tgr{ lpi "GT"uki pcrlpi . "kpenw lpi "papi gpqo le" cpf "i gpqo le"cevkpu0'Ku"cevkxk{ "ku"o qf wrcvgf "d{ "G4"y tqwi j "o wnr ng"o gej cpluo u."ngcf lpi "vq" dtgcu'ecpegt"egm'r tqrlhgtcvkp."kpxculqp."cpf "o gvcucuku0J gtg."y g'hqwpf "y cv'e/Ute'y cu"wkrl gf " d{ " G4" vq" kpf weg" cr qr vquku/tgrvgf " r cvj y c{u" kpenw lpi " qzkl cvkxg" utguu" cpf " kphco o cvqt { " tgr qpugu"ge0G4"ecwugf "o kqej qpf tkcn'f {uhwpevkqp"y tqwi j "f kutw lpi "o kqej qpf tkcn'o go dtcpg" kpgi tkf{."hceklcvlpi "y g'vcpugcevkqp"qh'e{vqej tqo g"e"ltqo "y g'o kqej qpf tkc"kpq"y g"e{vquqn" cpf " kpf wekpi " qzkl cvkxg" utguu0' CR/3" ku" qhvgp" eqpukf gtgf " cu" c" pwerget " f gekukp/o cngt " y cv' f gyto kpgu"rlhg"qt"f gcj "egm'hcvgu"lp"tgr qpug"vq"gz vcegmwret"uuko wrk0'Dqj "GT"cpf "CR/3"ctg" pwerget"vcpuetk vlqp"lcevqtu0'Y g"dgrkxg"y cv'y g"tqrg"qh'CR/3"lp"cr qr vquku"uj qwf "dg"eqpukf gtgf " y kj lp"e"eqo r ngz"pgwy qtn'qh"pwerget"lcevqtu"y cv'tgr qpf "uko wncpgqwn{ "vq"e"y kf g"tcpi g"qh" uki pcn'vcpuf wevkqp"r cvj y c{u"vki i gtgf "d{ "gustqi gp0'CR/3"dgrpi u"vq"dcuke'tgi kq/rgwelkg"l kr gt" *d\ Æ+" r tqvklp" y j lej " ecp" kpgtcev" y kj " pwerget " r tqvklp" P H/nerr c" D" *33+" cpf " qj gt" d\ Æ" vcpuetk vlqp"lcevqtu"uwej "cu"ETGD3"*37-0'K'y kn'dg"e"ej cnngpi g"vq"kf gpvkl{ "j qy "GT"tgi wrcvgu"qt" cuuqekcvgu"y kj "CR/3"y tqwi j "qj gt"pwerget"r tqvklp"vq"vki i gt"cr qr vquku/tgrvgf "i gpgu"lp"mipi / vgo "gustqi gp"f gr tkxgf "egm0""

"

"

VCUM'4<'*1 WLqtf cp+/'Vq'gnwelf cvg'vj g'b qngewret'b gej cpluo 'qh'G4'kpf wegf 'lwt xkxclcpf " cr qr vquku' lp" dtgcu' ecpegt " egm1' t gukwcpv' vq" gkx gt " UGTO u' qt " mpi /vgt o " gustqi gp" f gr t kcvlqp0'

Vcunl'4d/5<'*Ugpi wr v"cpf "Lqtf cp+'ó"Vq'gnwelf cvg'vj g'o gej cpluo u'qh'G4/lpf gr gpf gpv' i tqy vj 'qh'ldtgcuv'ecpegt 'egm1'b lo lenlpi 'btqo cvug/lpj kdkqt 't gukwcpv'f j gpqv' r gu'

Vcunl'4d/5<'*Ugpi wr v"cpf "Lqtf cp+/'Uwf lgu'ecttkgf "qww'd{ "F t0'Uwtqlggv'Ugpi wr v"lp"vj g"Lqtf cp" rddqtcvqt { "cv'I gqti gxy p"Wpkxgtuky/ "

"

Tqrg'qh'eO [E'cu'e'Etklecn'F gygo lpcpv'qh'Gustqi gp/kpf gr gpf gpv'I tqy vj 'qh'Gustqi gp/ F gr t kcvlqp"TGukwcpv'Dtgcuv'Ecpegt 'Egmi'"

Kpt qf wevkqp<'

Ctqwpf "ugxgpv{ "r gtegpv'qh'cm'pgy n{ "f kci pqugf "dtgcu'ecpegtu"gzr tguu"gustqi gp"tgegr vqt" crr j c" *GT +."kpf kecvlpi "f gr gpf gpeg"qp" gustqi gp"lqt"r tqrlhgtcvkp"qh"y g"GT "r quklxg"dtgcu' ecpegt"egm0'Ctqo cvug"lpj kdkqtu"*CK+."y j lej "r txxgpv'y g"u{pyj guku"qh'r gtr j gtrn'gustqi gp."ctg" wugf "gz vgpukxgn{ "vq"tgcv'GT "r quklxg."dtgcu'ecpegt"lp"r quwo gpqr cwucn'r cvkpw0J qy gxgt."mipi /

vgto "tgcvo gpv'y kj "CKku"cuuqekcvf "y kj "f kugcug"tgewttgpeg"f wg"vq"ces vkt gf "tgukncpegŒVj g" tgukncpv'dtgcuv'ecpegt"d{/r cuugu"vj g"f gr gpf gpe{ "qp"gutqi gp"cpf "eqpvkpwg"vq"r tqnhtcvg"kp"cp" gutqi gp/htgg"gpvktqpo gpŒVj gtghqtg."gnwelf cvkpi "vj g'o qrgewrct"f gvgto kpcpv'tgur qpukdrg"ht"vj g" i tqy vj "qh"vj g"C Kt gukncpv'dtgcuv'ecpegt"egmu"lp"cdugpeg"qh"gutqi gp"ku"vj g"ng{ "vq"kf gpvh{ "pqxgn" vj gter gwle"vcti guŒC"tgegpv'tgr qtvj cu'uwi i guvgf "cevkxcvgf "eO [E"i gpg'uki pcwtg"cu'y gmi'cu"j ki j " eO [E"r tqvqk"ngxgn"ku"cuuqekcvf "y kj "j ki j "wo qt"egm'i tqy vj "kp"C Kt gukncpv'dtgcuv'ecpegt" r cvkpvu"*38+Œ"Cpqvj gt"uwf { "j cu"cnq"tgr qtvf "vj cv"eO [E"o c{ "r nc{ "xgt { "ko r qtwcpv"tqrg"kp" o gxcvckle"dtgcuv'ecpegt"y kj "r qqt'r tqi pquku"*39+Œ

Kp"qwt'ncd"y gj' cxg'f gxgnr gf "c"o qf gn'ht"C Kt gukncpv'dtgcuv'ecpegt"egmu'd{ "i tqy kpi "vj g" GT "r qukkxg."ŒEHŒ"dtgcuv'ecpegt"egmu"lp"gutqi gp"f gr tkxgf "eqpf kkpqu"ht"qxgt"c" { gct."mpqy p" cu'ŒEHŒ<ŒE"egmuŒVj gug"egmu'ecp"i tqy "wpf gt"gutqi gp"f gr tkxgf "eqpf kkpqu"kp'xkt q"cu'y gmi'cu"kp" xkxq"cu"z gpqi tchv'wo qtu"lp"qxctkgevqo k gf."cvj { o le"o legŒKp"vj ku"ugevkp"y g"j cxg"uwf kgf "vj g" tqrg"qh" eO [E" qpeqi gpg" kp" gutqi gp/kpf gr gpf gpv" i tqy vj " wukpi " vj g" ŒEHŒ<ŒE" egmu." cpf " f gvgto kpgf "vj g"r quukdrg"wr /utgco "tgi wrvqt { "hcevqtu"tgur qpukdrg"ht"vj g"gzr tgukqp"qh"eO [E" o TPC"kp"vj gug"egmuŒ

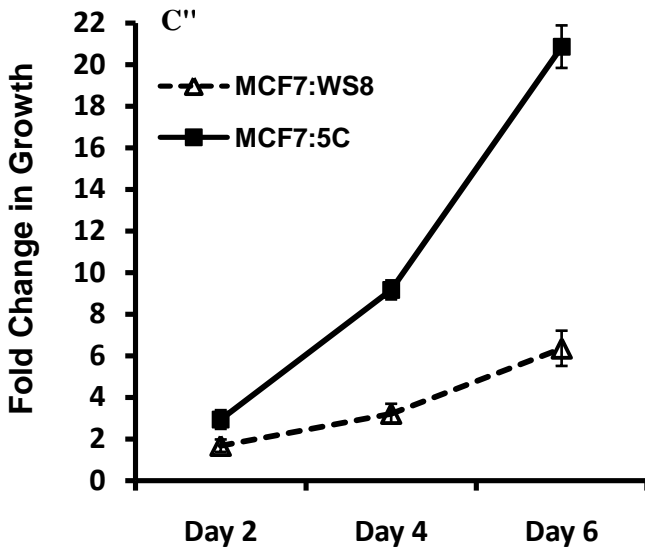
Y qtniCeeqo r ikuj gf <'

Gutqi gp"lpf gr gpf gpv"i tqy vj "qh'ŒEHŒ<ŒE"egmu'in vitro"cpf "in vivo"eqo r ctgf "y kj " r ctgpcvriŒEHŒ<Y U: 'egmu'

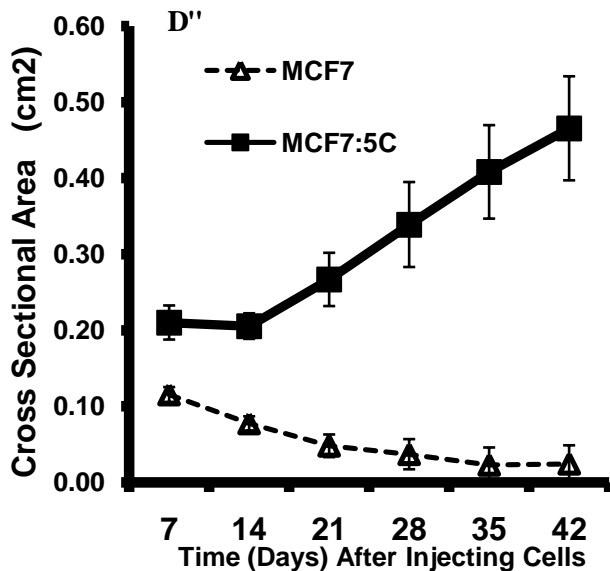
Y g'htuv'eqphko gf "vj g"gutqi gp/kpf gr gpf gpv"i tqy vj "qh'vj g"ŒEHŒ<ŒE"egmu"cpf "eqo r ctgf "k'y kj " vj g"r ctgpcvriŒEHŒ<Y U: "egmuŒVj g"i tqy vj "qh'ŒEHŒ<ŒE"egmu"kp'xkt q"y gtg"cv'ngcu'6/hqrf "j ki j gt" vj cp"vj g"r ctgpcvriŒEHŒ<Y U: "egmu"*Hki Œ35C+"lp"cdugpeg"qh"gutqi gpŒP gzv."y g'lpvgnvki cvgf "vj g" ur qpvcpgqwu."gutqi gp/kpf gr gpf gpv"i tqy vj "qh'ŒEHŒ<ŒE"egmu"kp'xkxq"d{ "kplgevki "7"o knkqp"egmu" dkrcvgtcm{ "lp"2Œ"o n'o kzgf "y kj "i tqy vj /hcevqt"tgf wegf "o cvki gn"lp"3<3"tcvkq"lp"qxctkgevqo k gf." pwlpw'o legŒŒEHŒ<Y U: "egmu"y gtg"cnq" wugf "cu"eqpvqtqfŒVwo qt"o gcuwtgo gpw"y gtg"vcnrgp" y ggm{ "uactvki "qpg"y ggm'r quv'kplgevkiŒCu"gxkf gpv'htqo "vj g"tguvnu"*Hki Œ35D+"ŒEHŒ<ŒE"egmu" hqto gf "vj g"zgpqi tchv'wo qtu"cpf "eqpukvgnv{ "i tgy "qxgt"ugxgp"y ggm'r gtlkf."y j gtgcu"vj g"r ctgpcvriŒEHŒ<Y U: "egmu"fkf "pqv'htqo "cp{ "wo qt"y j cvuqgxgtŒVj ku"guvdrkuj gf "vj cv'ŒEHŒ<ŒE"egmu'ecp" ur qpvcpgqwu{ "i tqy "kp'xkt q"cu'y gmi'cu"kp'xkxq"lp"cdugpeg"qh"cp{ "gutqi gpŒ"

"
"
"
"
"
"
"

Hli wt g'35"



Hli wt g'350'Gut qi gp"lpf gr gpf gpv'i t qy vj " qh' O EHŒ7E" egm0' C0' Ur qpvcpgqwu." gutqi gp/kpf gr gpf gpv'i t qy vj "qh'O EHŒ7E U: " qt" O EHŒ7E" egmu" qxgt" c" ulz" f c{ " r gtlkf 0' Ctqwpf " vgp" vj qwucpf " egmu" y gtg" r rvcgf " kp" gcej "y gmi'qh'46" y gmi' r rvcgf "cpf" vj g"i t qy vj " y cu" o qpkqtgf " qxgt" ulz" f c{ " r gtlkf 0' Vj g" F P C"eqpvgpv'kp" gcej "y gmi' y cu'o gcuwtgf "cu" c" uwtqi cvg" qh" i t qy vj 0' D0' Hqto cvkqp" cpf " i t qy vj " qh" zgpqi tchv" wo qtu" qh' O EHŒ7E" egmu"kp"cdugpeg"qh'gutqi gp"eqo r ctgf "y kj " O EHŒ7E U: "egmu"qxgt" c" r gtlkf "qh'ulz"y ggmu0' Ctqwpf "7"o krlqp"egmu"o kzgf "y kj "o cvki gni" y gtg" kplgevgf " dkrcvgcm{ " kp" gcej " qxctlgcvqo k gf "pwf g"o leg"cpf"vj g"i t qy vj "qh' vj g"wo qtu"y gtg"o qpkqtgf "ltqo "ltuv"y ggmi' vj tqwi j " y ggmi' 90' P kpg" wo qtu" y gtg" o qpkqtgf "hqt"O EHŒ7E"cpf "7"wo qtu"y gtg" o qpkqtgf "hqt"O EHŒ7E U: "zgpqi tchv0"



Dcucnlgzr tguakp'lgxgn'qhl'eO [E'b TPC'cpf 'rtqvglp'lp'O EHŒ7E U: 'cpf 'O EHŒ7E'egmi'

eO [E"ku"cp"qpeqi gpg"y j lej "ku"f g/tgi wrcvgf "kp"o cp{ "ecpegtu"cpf "eq/tgrcvu"y kj "r qqt" r tqi pquku"*3: +cpf "r rc{ "u"etwecn'tqng"lp"egmi'e{ eng"r tqi tguakp"cpf "tgi wrcvpi "xctlgv{ "qh"i gpgu" *3; 40'K"ku"cnq" c"xgt{ "y gmi'ej ctcevgtk gf "gutqi gp/tgi wrcvgf "i gpg"kp"GT "r qukkxg"dtgcuv'ecpegt" egmu"*42."43-0'Y g"vj gtghqtg" gxcnrcvgf "vj g"dcucn'gzr tguakp"ngxgn'qh'eO [E"i gpg"kp"gutqi gp/ f gr tlxgf " tguakcpv" O EHŒ7E" egmi" cpf " eqo r ctgf " y kj " vj g" r ctgpcn' O EHŒ7E U: " egmu0' kpvgtgukpi n{."y g'hqwpf "vj cv'vj g"dcucn'gzr tguakp"qh'eO [E"o TPC"cu"y gmi'cu'vj g'r tqvklp"y cu'cv' ngcu'5/6'hqr" j ki j gt"lp"O EHŒ7E"egmu"cu'eqo r ctgf "vq"r ctgpcn'O EHŒ7E U: "egmi"*Hli 036C"cpf " 36D-0'Vj g"dcucn'gzr tguakp"qh'GT "r tqvklp"y cu'cnq"j ki j gt"lp"vj g"O EHŒ7E"cu'eqo r ctgf "vq" O EHŒ7E U: "egmu0"

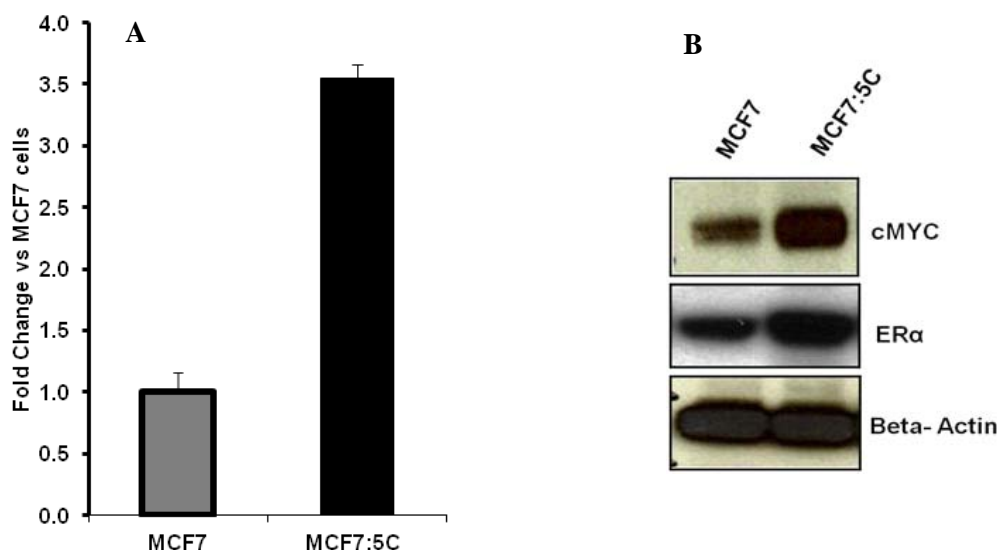
Figure 14

Figure 14. Basal expression of cMYC in MCF7:WS8 and MCF7:5C cells. **A.** mRNA levels of cMYC in MCF7:WS8 and MCF7:5C cells. Transcripts levels were measured using real-time PCR using 36B4 as an internal control. Data is re-presented in terms of fold difference as compared to MCF7:WS8 cells. **B.** Protein levels of cMYC, ERα and Beta-actin protein levels were measured as loading control.

Effect of pharmacological inhibition of cMYC by 10058-F4 compound on the estrogen independent growth of MCF7:5C cells

To understand the functional relevance of the cMYC over-expression in the MCF7:5C cells, we investigated the role of cMYC in estrogen-independent growth of MCF7:5C cells. We treated the MCF7:5C cells and MCF7:WS8 cells with a cMYC inhibitor, 10058-F4, in increasing concentration. We found that MCF7:5C cells were drastically more sensitive to 10058-F4 mediated inhibition of spontaneous growth than MCF7:WS8 cells after 2 and 4 days of treatment (Fig. 15A and 15B).

The actively proliferating cells. The cMYC inhibitor, 10058-F4, remarkably decreased the growth of MCF7:5C cells in a dose dependent manner in the MCF7:5C treated cells, but the effect on MCF7:WS8 was very minimal (Fig. 15C and 15F). The growth of MCF7:5C cells were decrease with 30uM of the inhibitor in MCF7:5C cells as compared to only 10% in parental cells suggesting that cMYC plays a critical role in estrogen-independent growth of MCF7:5C cells.

Figure 15

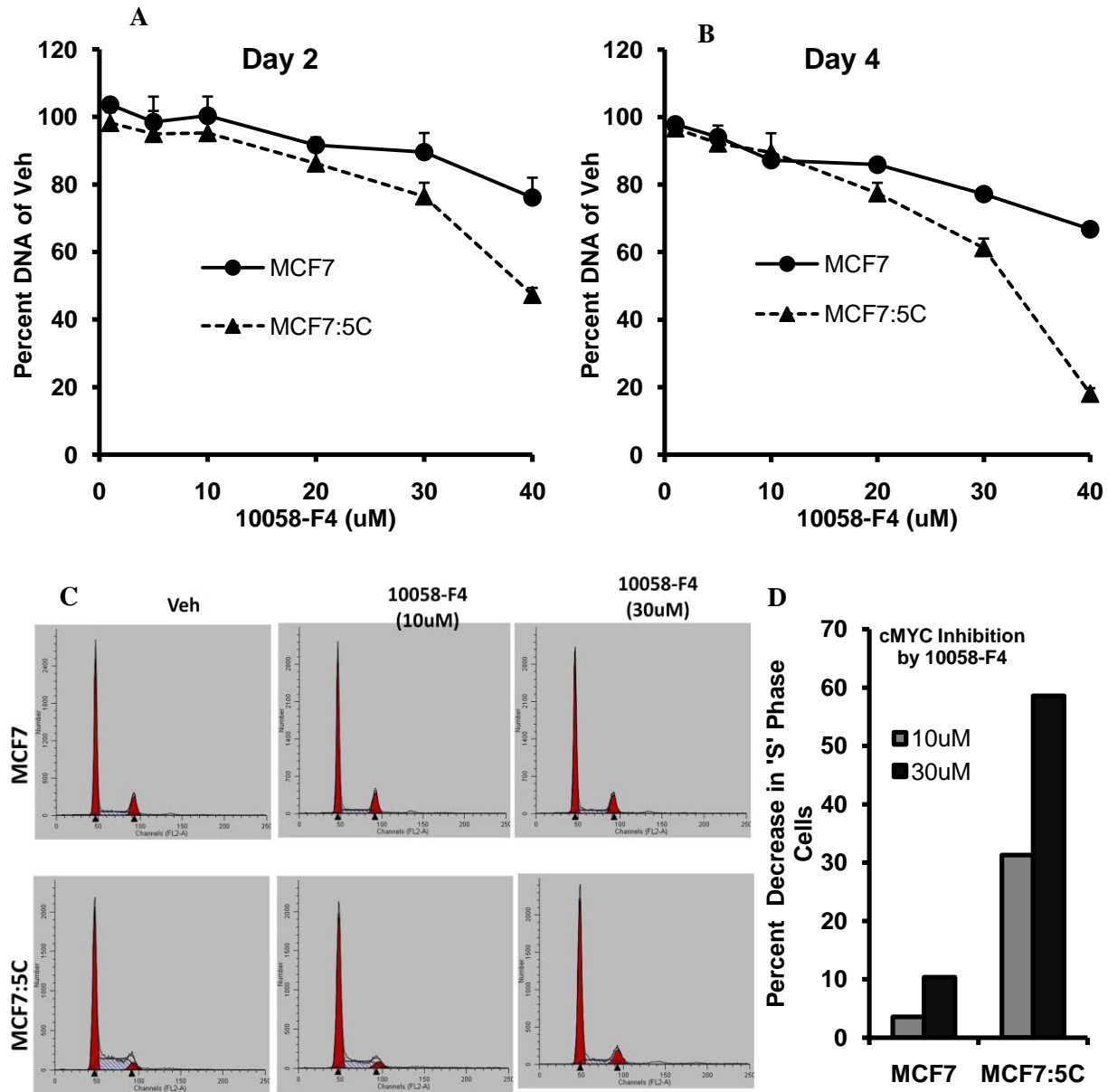


Figure 15. Effect of cMYC inhibitor (10058-F4) on spontaneous growth of MCF7 and MCF7:5C cells. A. Comparison of growth inhibition of MCF7 and MCF7:5C cells by cMYC inhibitor 10058-F4 in a four day growth assay. **B.** Dose dependent growth inhibition of MCF7:5C cells using cMYC inhibitor over a six day period. **C.** Dose dependent growth inhibition of MCF7:5C cells using cMYC inhibitor over a six day period with parental counterpart MCF7 cells. **D.** Dose dependent growth inhibition of MCF7:5C cells using cMYC inhibitor over a six day period with parental counterpart MCF7 cells.

Effect of complete anti-estrogen, fulvestrant, on growth and cMYC levels by 10058-F4 in MCF7:5C cells

Next, we evaluated the effect of the complete anti-estrogen, fulvestrant, on the growth and cMYC levels of MCF7:5C cells to understand the role of un-*rk* *cpf* *gf* "GT" "k" "v" *gug* "egm" "Y" *g* checked the growth by treating the cells with 1uM of fulvestrant over a six day period (Fig. 16A), and found that fulvestrant inhibits the estrogen-independent growth of MCF7:5C cells by around 50 percent. Interestingly, cMYC mRNA as well as the protein levels were also decreased by similar extent after 24 hrs of treatment (Fig. 16B and 16E) *0Cu* *gzi* *gevgf* "GT" "r" *tqvgk* "rgxgn" were undetectable after fulvestrant treatment. This suggests that un-*rk* *cpf* *gf* "GT" "r" *tqvgk* "o" *c* { "dg" partially responsible for the over-expression of cMYC protein in MCF7:5C cells.

Figure 16

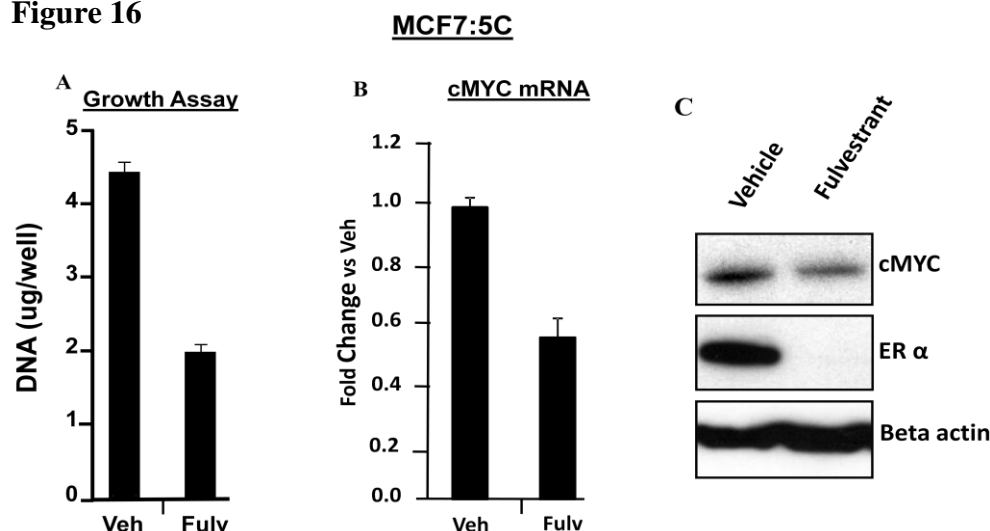


Figure 16. Effect of fulvestrant (Fulv) on growth, cMYC mRNA and protein levels in MCF7:5C cells as compared to vehicle. A. Growth of MCF7:5C cells in presence of 1uM fulvestrant (Fulv) over a six day period. **B.** Levels of cMYC mRNA after 24 hrs treatment with 1uM fulvestrant (Fulv) **C.** Protein levels of cMYC and estrogen receptor (ER) after treatment with 1uM fulvestrant for 24 hrs.

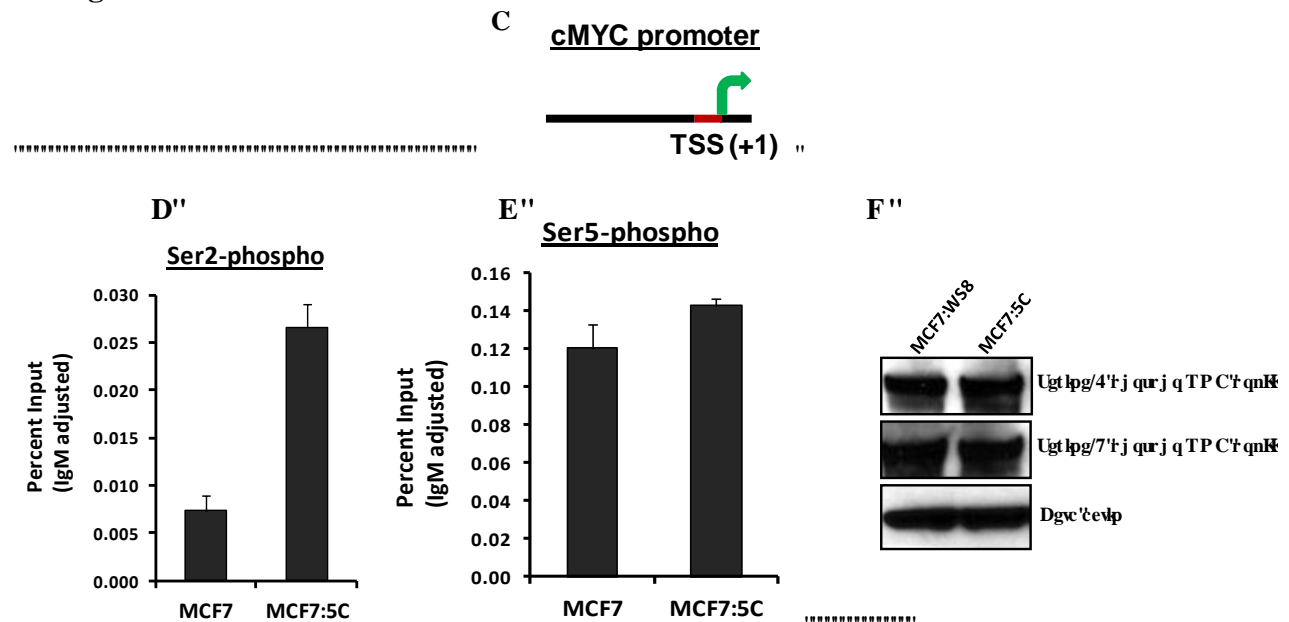
Recruitment of phosphorylated serine 2 and serine 5 RNA polymerase-II at the proximal promoter of cMYC gene in MCF7:WS8 and MCF7:5C cells

To study the up-stream factors involved in the high transcriptional activity of cMYC gene in MCF7:5C cells we evaluated the recruitment of the phosphorylated-serine 2 and phosphorylated serine-5 RNA polymerase-II at the proximal promoter of the cMYC gene (Fig. 17A), using chromatin immune-precipitation assay. RNA polymerase-II is a multimeric protein complex responsible for the transcription of the genes. However, the RNA polymerase-II needs to be phosphorylated at serine-5 of the carboxyl-terminal domain (CTD) for the initiation of the transcriptional process and phosphorylation of the serine-2 of the same CTD is required for the elongation of the transcription (22, 23).

Our results revealed that phospho-serine-5 as well as phospho-serine-2 RNA polymerase-II was constitutively recruited at the promoter of the cMYC gene in MCF7:5C cells (Fig. 17B

cpf "39E-0' k" eqo r ctluqp. "k" vj g" r ctgpcn' O EHŒY U: "egm." tgetwko gpv' qh' rj qur j q/ugt kpg/4" TPC" r qn' o gtcug/ K' y gtg' cv' hcu' 5/hqf' 'guu' vj cp' vj g' O EHŒ7E" egm' 0' Qh' pqvg. "vj g' tgetwko gpv' qh' rj qur j q/ugt kpg/7" TPC" r qn' o gtcug/ K' k' dqvj "vj g' egm' f k' "pqv' uj qy "cp{ "f k' hgt gpeg" *Hk' 0' 39E-0' Y g' cnuq' ej gengf "vj g' ngxnu' qh' rj qur j q/ugt kpg/7" cpf "rj qur j q/ugt kpg/4" TPC" r qn' o gtcug/ K' k' vj g' vqcn' r tqvgk' n' ucvgu' qh' vj gug' egm' cpf "hwpf "pq' f k' hgt gpegu" *Hk' 0' 39F-0' Vj ku' kpf kecvgf "vj cv' vj g' f k' hgt gpv' cn' tgetwko gpv' qh' rj qur j q/ugt kpg/4" TPC" r qn' o gtcug/ K' cv' vj g' r tqo qvgt "qh' eO [E" k" O EHŒ7E" egm' o c{ "dg' f wg' uqo g' ej tqo cvk' ej cpi gu' cv' vj g' r tqo qvgt' ngxnu' y j lej "o gt ku' hmt vj gt" kpxgu' k' cvk' p0'

Hk' wt g' 39"



Hk' wt g' 390' Tgetwko gpv' qh' ugt kpg/4/ rj qur j qt { rvgf "cpf "ugt kpg/7/ rj qur j qt { rvgf "TPC" r qn' o gtcug/ K' cv' vj g' eO [E' r tqo qvgt 0' C0' F k' tco "f gr kexpi "vj g' eO [E' r tqo qvgt' y kj "vj g' gvtqi gp' t gur qpukxg' grgo gpv' k' tgf +cpf "ku' vcpuetkr vkp' uctv' uksg" *VUU+ D0' Tgetwko gpv' qh' ugt kpg/4/ rj qur j qt { rvgf "TPC" r qn' o gtcug/ K' cv' vj g' eO [E' r tqo qvgt "k" O EHŒ" cpf "O EHŒ7E" egm' 0' E0' Tgetwko gpv' qh' ugt kpg/7/ rj qur j qt { rvgf "TPC" r qn' o gtcug/ K' cv' vj g' eO [E' r tqo qvgt "k" O EHŒ" cpf "O EHŒ7E" egm' 0' Vj g' f cv' ku' tgr tgu' gvgf "cu' r gtegpv' kpr w' cf lwvgf "hqt' eqp' t qn' o qwug' K O " cpv' kdf { 0' F0' Ngxnu' qh' vqcn' ugt kpg/4" rj qur j qt { rvgf "cpf "ugt kpg/7" rj qur j qt { rvgf "TPC" r qn' o gtcug/ K' k' O EHŒY U: "cpf "O EHŒ7E" egm' 0"

Tqrg' qh' e{ enlp' f gr gpf gpv' nlp' cug'; *EFM; +lp' gvtqi gp/ kpf gr gpf gpv' i tqy vj "qh' O EHŒ7E" egm' cu' eqo r ctgf "vq' r ctgpcn' O EHŒY U: "egm' "

P gzv. " y g' j { r qv' guk' gf " vj cv' k' ugt kpg/4" rj qur j qt { rvkqp' qh' TPC" r qn' o gtcug/ K' ku' tgur qpukdrg' hqt" vj g' vcpuetkr vkpcn' qxgt/ gzt' gukqp' qh' eO [E" vj gp' dmenkpi " vj g' ugt kpg/4" rj qur j qt { rvkqp' uj qwf "cnuq' k' j kdk' vj g' gvtqi gp/ kpf gr gpf gpv' i tqy vj "qh' vj g' O EHŒ7E" egm' 0' k' vj ku' f k' gevkp' y g' htu' vj gengf "vj g' ngxnu' qh' e{ enlp' f gr gpf gpv' nlp' cug/; *EFM; +y j lej "ku' c" o clqt" nlp' cug' tgur qpukdrg' hqt' vj g' rj qur j qt { rvkqp' qh' ugt kpg/4" qh' TPC" r qn' o gtcug/ K' *45-0' k' vgt gukpi n' . " vj g' vqcn' EFM; " ngxnu' cu' y gni' cu' vj g' rj qur j qt { rvgf "EFM; " ngxnu' y gtg' uki p' k' h' c' p' v' n' " j k' j gt "k" O EHŒ7E" egm' cu' eqo r ctgf "vq" O EHŒY U: "egm' *Hk' 0' 3: C+ y j lej "uwi i guvgf "vj g' tgcup" hqt"

increased serine-2 phosphorylation in the MCF7:5C cells. Thereafter we used a CDK9 inhibitor, CAN 508 (also known as CDK9 inhibitor II), to block serine-2 phosphorylation of RNA polymerase-II and evaluated its effect on growth of MCF7:WS8 and 5C cells. Indeed, we found that the growth of MCF7:5C cells were selectively more inhibited by the CDK9 inhibitor in a dose dependent manner than the parental MCF7:WS8 cells (Fig. 18B). A growth curve using two different concentrations of the CDK9 inhibitor over a six day period revealed that 30uM of CAN508 was able to completely block the estrogen-independent growth of the MCF7:5C cells (Fig. 18C). These data supports our hypothesis that inhibition of CDK9 in MCF7:5C cells suppresses the estrogen-independent growth, most likely by down-regulating the levels of cMYC by blocking the serine-2-phosphorylation of RNA polymerase II in these cells.

Overall, this section reports a novel mechanism by which cMYC transcripts are regulated by estrogen deprivation and elucidates the upstream factors involved in driving the over-expression of the cMYC oncogene which is responsible for the estrogen-independent growth of the MCF7:5C cells, which mimics the aromatase-resistant breast cancer cells.

Figure 18

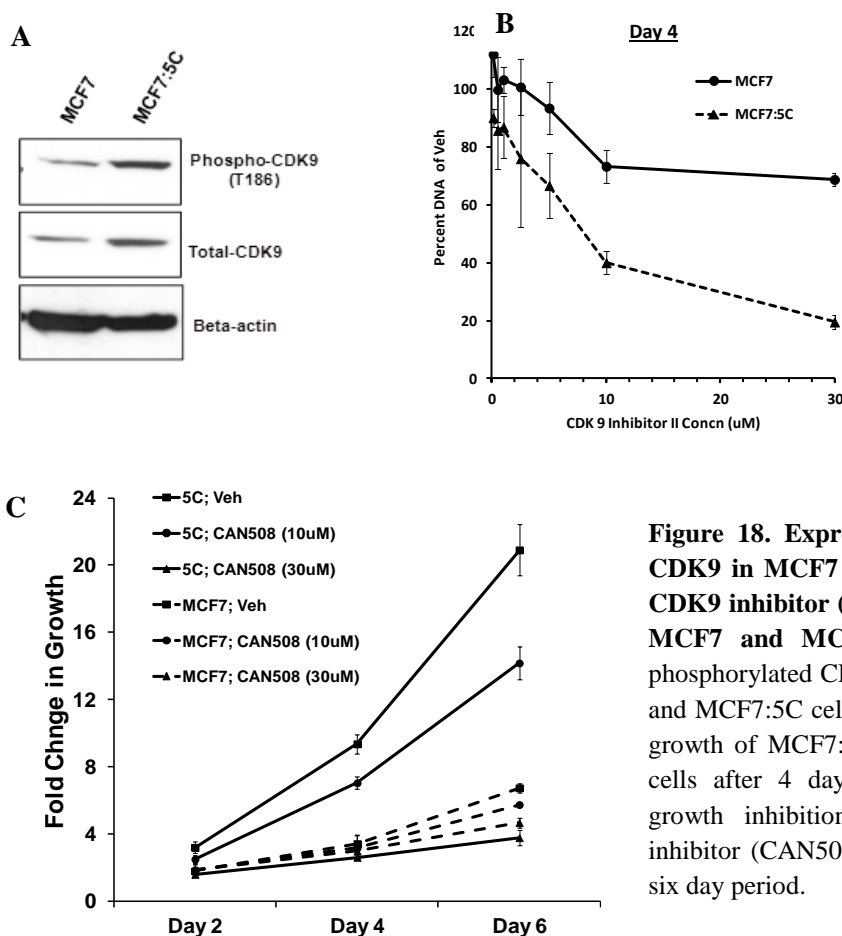


Figure 18. Expression of phosphorylated and total CDK9 in MCF7 and MCF7:WS8 cells and effect of CDK9 inhibitor (CAN508) on spontaneous growth of MCF7 and MCF7:5C cells. A. Protein level of phosphorylated CDK9 (T186) and total CDK9 in MCF7 and MCF7:5C cells. **B.** Effect of CDK9 inhibitor on the growth of MCF7:5C cells as compared to MCF7:WS8 cells after 4 days of treatment. **C.** Dose dependent growth inhibition of MCF7:5C cells using CDK9 inhibitor (CAN508) compared with MCF7 cells over a six day period.

VCUM4<"*I WLqtf cp+/'Vq'gñwelf cwg'vj g'b qñgewrct 'b gei cpluo 'qh'G4'lpf wegf 'lwt xkcnlcpf " crqr vquku' lp" dt gcw' ecpegt ' egmi' t gukwcpv' wq' gkñ gt " UGTO u' qt " mpi /vgt o " guntqi gp" f gr t kcvkp0'

Vcunl'4d/6<"*Ugpi wr w."Qdkqt cñ "cpf "Lqtf cp+/'ó"Vq"eqplkt o "cpf "xcñf cwg" f gxgnr lpi " r cvj y c{ u'qh'G4/lpf wegf 'dt gcw'ecpegt 'egmiltwt xkcnlcpf 'cr qr vquku0'

Vcunl'4d/6<"Ugpi wr w."Qdkqt cñ "cpf "Lqtf cp+/'Uwf kgu'ecttkgf "qw'd{ "F t0'Uwtqlggv"Ugpi wr w"cpf " F t0'Kgf lpy c'Qdkqt cñ 'lp'vj g'Lqtf cp'rdqtcvqt { "cvI gqti gvy p'Wpkxgtukv " "

F gclj gt lpi "vj g"O gei cpluo "qh'Cevkqp"qh'Dkñ j gpñl'cpf "Dkñ j gpñl'C"lp"I t qy vñ "cpf " Cr qr vquku'qh'Dt gcw'Ecpegt 'Egmi'O gf kcvf 'd{ 'Guntqi gp'Tgegr vqt 'Cñ j c'"

Kpvt qf wekqp<'

Guntqi gp'tgegr vqt "cñ j c"*GT +o gf kcvu"ku"cevkp"d{ "dkpf lpi "v"ku"eqi pcvg'ñi cpf u"cpf " hñpevkqp"cu" c'ñi cpf /cevxcvqf ö"tcpuetr vqp"ñcevqt"lp"egmi"cpf "vkuwgu0'Cr ctv'ltqo "ku"pcwtcn' ñi cpf u."ugxgtcn'utwewtcm{ "uko kñt"eqo r qwpf u"ecp"dkpf "v"GT "cpf "vj wu"ecp"ñpevkqp"cu"ku" ñi cpf "*46+0J qy gxgt."f gr gpf lpi "wr qp'vj g'ej go kcn'utwewtgu'qh'vj gug'ñi cpf u'vj g{ "ecp"ñpevkqp" cu"cp"GT "ci qpkuv"qt"cu"cp"cpñi qpkuv"qt"gxgp" c"r ctvñl'ci qpkuv"cpñi qpkuv0'O cp{ "ej go kcn{ " f kxgtug"eqo r qwpf u"ecp"ñpevkqp"cu"cp" guntqi gp"qp"xctkqu"cuuc{ u0'Dtqcf ñ. "vj gug" guntqi gple" eqo r qwpf u"ecp"dg"enñkñgf "cu"enñu"K'cpf "enñu"K'f gr gpf lpi "wr qp'vj gk"r ñpct"qt"pqp/r ñpct" *cpi wrct+"ej go kcn'utwewtgu."t gurgvñgn{ "*47+0F kñgt gp'ñi cpf u"ecp"dkpf "v"vj g"uco g"eqtg'qh" vñ g"ñi cpf "dkpf lpi "f qo clp"*NDF +qñ'vj g"GT "r tqvñp"dw"ecp"gxqng"f kñkpev'y tgg/f ko gpukqpcñ' eqphqto cñqp"qh'vj g"ñi cpf gf /tgegr vqt"eqo r ngz"y j kñ "ecp"gkñ gt "kpvtcev'y kj "vj g"eq/cevxcvqtu" qt" vñ g" eq/tgr tguuqtu" *eqngvñgn{ " npqy p" cu" eq/tgi wrvqtu+" cv" vñ g" r tqo qvgtu" qñ" guntqi gp" t gurgpukxg"i gpgu"*48+0'E qpugs vgpñ. "vj ku"eqo r ngz"o qf wrvñu"vj g"tcpuetr vqpñ'cevñkñ{ "qh'vj g" xctkqu" guntqi gp/t gurgpukxg"i gpgu"cpf "gxgpwcm{ "f gvgto kpñu'vj g"qweqo g"qh'vj g"GT "f gr gpf gpv" r j { ukññi kcn'ñt gurgpugu'qh'c"r ctvñwrt "egm'qt "vkuwvñ{ r g0"

Vj g" o qñgewrct "dcuku" qñ" vñ ku" f kñgt gpñl' tgetvko gpv' qñ" vñ g" eq/tgi wrvqtu" j cu" dggp" cwtkdwgf "v"vj g"cdkñkñ{ "qh'vj g"ñi cpf gf /GT "v"tg/qlkpv'vj g"j gñk "34"qh'vj g"NDF "uq"vj cv'vj g" eqo r ngz"ecp"kpvtcev'y kj "vj g"eq/cevxcvqtu"cv'vj g"utwewtcn'kpwthceg"ñqto gf "d{ "J 5."J 6"cpf "J 7" j gñlegu."y j gp"GT "ku"dqwpf "v"cp"ci qpkuv"*39/dgvc" guntcf kññt "F GU+"*49."4: +dw'vj ku'kpvtcevqp" y kj "vj g"eq/cevxcvqtu"ku"eqo r ngvñ{ "dñqngf "y j gp"vj g"GT "ku"dqwpf "v"cpñi qpkuv."uwej "cu"6/ j { ftqz{/wco qz kñgp"*6QJ V+"*49+"qt"tcmz kñgp"*TCN+"*4: +0'kpvtgukñi ñ."y j gp"GT "ku"ñi cpf gf " y kj "cp"cpñi qpkuv"kv"ecp"pqy "kpvtcev'y kj "vj g"eq/tgr tguuqtu"cpf "ecp"kpñ kñkñ'vj g"tcpuetr vqpñ' cevñkñ{ "qh'vj g" guntqi gp't gurgpukxg"i gpgu"*42+0'Dguk gu'vj g"kpvtcevqp"qh'eq/tgi wrvqtu""y kj "vj g" ñi cpf gf "GT ". "vj g'gñgn'qh'eq/cevxcvqtu"cpf "eq/tgr tguuqtu"lp" c"i kxgp"egm'ecp"cnq" f gvgto kpñ'vj g" r j { ukññi kcn'ñt gurgpugu'vñ f kñgt gpñl'ñi cpf u'qh'GT " *42+0"

Uwf kgu"ltqo "qwt"ñd"j cxg"ñf gpñkñgf "vj cv'vj g"co kpñ"cekf "cur ctvñg"cv"573"r qukkqp"lp"vj g" j gñk "5qh"NDF "qh'vj g"GT "ku"etkñecm{ "ko r qtvcvñ'ñt"o clpvñlpi "vj g"kpñi tkñ{ "qh'cpñk guntqi gple" cevñkñ{ "qh'ngqz kñgp"*tcmz kñgp+"cpf "wco qz kñgp"*4: . "52+0Rt gñkñwññ. "ku"uñdñkñkñp"o wcvkp"vñ vñ tqukpñ"co kpñ"cekf "y cu" f gvgvñf "lp"qpñ"qh'vj g"z gpñi tch'wo qtu"uñk wñvñf "d{ "wco qz kñgp"lp"vj g"

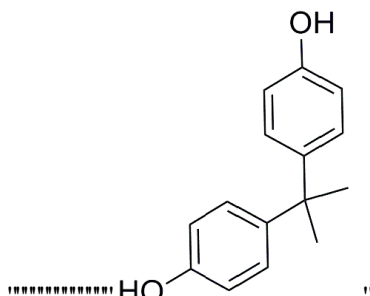
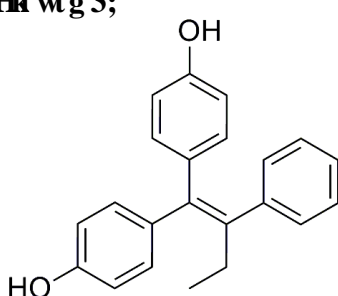
cvj {o le"o leg"*53-0'Hwtvj gt"lpxgunki cvkqpu"j cxg"tgxgcrgf "vj cv'ej cpi kpi "vj g"573"cur ctvcvg"co kpg" cekf "qh'vj g"GT "vq"i n{ekpg"*F 573I +cdqrkuj gu"vj g"gutqi gpke"ghgeV'qh'6QJ V'dw'f qgu"pqv'chgeV' gutcf kqn'cevkap"qp"VI H "i gpg"cevkcckap"lp"vj g"GT"pgi cvkxg"egmu"ucdn{"tcpuhevgf "y kj "gkj gt" y krf "v{r g"GT "qt"F 573I "o wcvgf"GT "54-0'Wulpi "vj gug"o qf gnu."gutqi gpu"y gtg"encukhgf "cu" gkj gt"v{r g"K'y j lej "j cxg"vj g'r rcpct"utwewtgu"qt"v{r g"KK'y j lej "j cxg"vj g"cpj wrct"qt"pqp/r rcpct" utwewtgu"*47."55-0'Kp"vj ku'ugevkap"y g'f gvgto kpgf "vj cv'vj g'tgs wkukg"eqphqto cvkqp"qh'vj g'rki cpf gf / GT "eqo r rnz"ku'f khtgvp'hqt"lpf welpi "i tqy vj "cpf "cr qr vuku"d{ "wulpi "vy q"gutqi gpke"eqo r qwpf u" dkur j gpqn'cpf "dkur j gpqn/C"qp"i tqy vj "qh'O EHŒ<Y U: "egmu"cpf "cr qr vuku"lp"mpj "vgto "gutqi gp" f gr tkxgf "O EHŒ<7E"egmu0'

Y qtmiCeeqo r rkuj gf <'

F khtgtpvkn'ghgeV'qh'Dkur j gpqn'cpf 'Dkur j gpqn/C'lp'lpf welpi 'cr qr vuku'lp'O EHŒ<7E'egmu' dw'pqv'i tqy vj 'lp'O EHŒ<Y U: 'egmu'

Dkur j gpqn"*DR+*"Hki 0'3; +c"vkr j gp{rgvj {rgpg"*VRG+ku"cnpy p"gutqi gpke"rki cpf "y j lej " ecp"lpf weg"i tqy vj "qh'vj g"GT "r qukkxg"dtgcuv'ecpegt"egmu"*56-0'Cpqvj gt"eqo r qwpf "y kj "uko krt" ej go lecn' utwewt g." dkur j gpqn' ŒC" *DRC+*"Hki 0' 3; +ku" cnuq" c" y gm' ej ctcevgtk gf " dw' y gcm' gutqi gpke"rki cpf 0"

Hki wt g'3; "



Hki wt g' 3; 0' Ej go lecn' utwewt gu' qh' Dkur j gpqn' *DR+* cpf " Dkur j gpqn/C" *DRC+0'

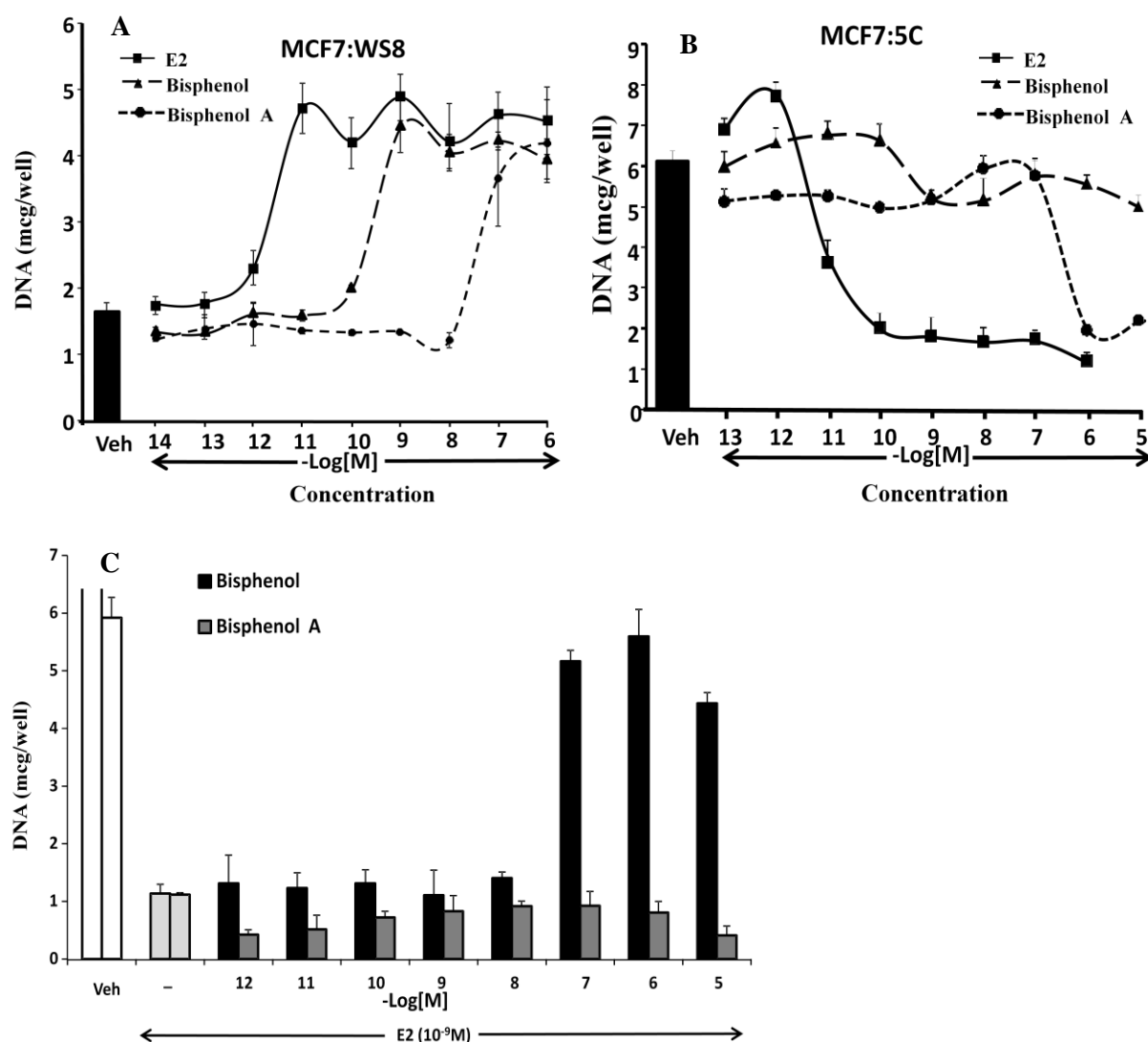
" Dkur j gpqn*DR+"

Dkur j gpqn/C*DR+"

J gtg"y g"gxcmvcvgf "vj g"cdkkrv{ "qh'vj gug"vy q"gutqi gpke"eqo r qwpf u"vq"lpf weg"i tqy vj "cpf " cr qr vuku" lp" O EHŒ<Y U: " cpf " O EHŒ<7E" egmu" tgr gevkxgn{." cu" dqvj " vj gug" tgr qpugu" ecp" dg" lpf weg" d{ "gutqi gp0Cu"gzr gewf ."DR"cu"y gm'cu"DRC"y cu'cdrg"vq"lpf weg"f qug"f gr gpf gpv'i tqy vj " lp"vj g"O EHŒ<Y U: "egmu"*Hki 0'42C-0'DRC"y cu'ngu'r qvgpv'eqo r ctgf "vq"DR"cu"o czko cn'i tqy vj " y cu'cej kxgf "d{ "DR"cv'32⁷ O "eqpegpvcvkqp"cu'eqo r ctgf "vq"32⁸O "hqt"DRC0Cu"cu'eqo r ctgkqp" 39 /gutcf kqn*G4+y cu'cdrg"vq"lpf weg"o czko cn'i tqy vj "cv'32³³O "eqpegpvcvkqp"lp"vj g"O EHŒ<Y U: " egmu0'Kpvtgukpi n{." o ctngf "eqpvcuv"y cu'qdugtxgf "lp"lpf welkap"qh'cr qr vuku"d{ "DR"cpf "DRC"lp" O EHŒ<7E"egmu"y j lej "vpf gti q"cr qr vuku"y kj "G4"tgcvto gp0DRC"y cu'cdrg"vq"lpf weg"cr qr vuku"vq" vj g"uco g"gzvgpv'cu"G4"lp"vj gug"egmu"cv'c"j ki j gt"*32⁸O +eqpegpvcvkqp"*Hki 0'42D+cu'eqo r ctgf "vq" G4"y j lej "cej kxgf "vj g"o czko cn'cr qr vuku"cv'32³²O 0'J qy gxgt."DR"hcrgf "vq"lpf weg"cr qr vuku" gxgp"cv'32⁷O "eqpegpvcvkqp"*Hki 0'42D+0Y g"hwvj gt"lpxgunki cvgf "vj cv'kh"DR"y cu'cewcm{ "dkpf kpi " vq"vj g"GT "lp"vj g"O EHŒ<7E"egmu"d{ "tgcvkpi "vj gug"egmu"y kj "DR"lp"eqo dlpcvkqp"y kj "32⁷ O "qh' G40DR"y cu'cdrg"vq"dmqen'vj g"ghgeV'qh'G4"lp"vj g"O EHŒ<7E"egmu"*Hki 0'42E+lp"cu" f qug" f gr gpf gpv' o cppgt"lpf lecvkpi "vj cv'DR"y cu'cewcm{ "dkpf kpi "vq"vj g"GT . "vj wu'lpj kdkkpi "vj g"G4"cevkap0Qp"vj g"

other hand, BPA was not able to block the effect E_2 action (Fig. 20C). In addition, we also show that the estrogenic effect of BPA ($10^{-6}M$) in inducing apoptosis in MCF7:5C cells was completely blocked by BP ($10^{-6}M$) as well as $10^{-6}M$ of 4-hydroxy tamoxifen (4OHT) (Fig. 20D). These experiments established that unlike BPA and E_2 , BP was not functioning as an estrogen in inducing apoptosis while both compounds (BPA and BP) were equally estrogenic in inducing i tqy vj "kp"OEH9<Y U: "egmu0Vj ku"engctn{"uwi i guvgf "f kht gpvkn"tgs wkt go gpv"qh"GT "o gf kcvgf " molecular action to achieve two distinct physiological responses in the breast cancer cells.

Figure 20



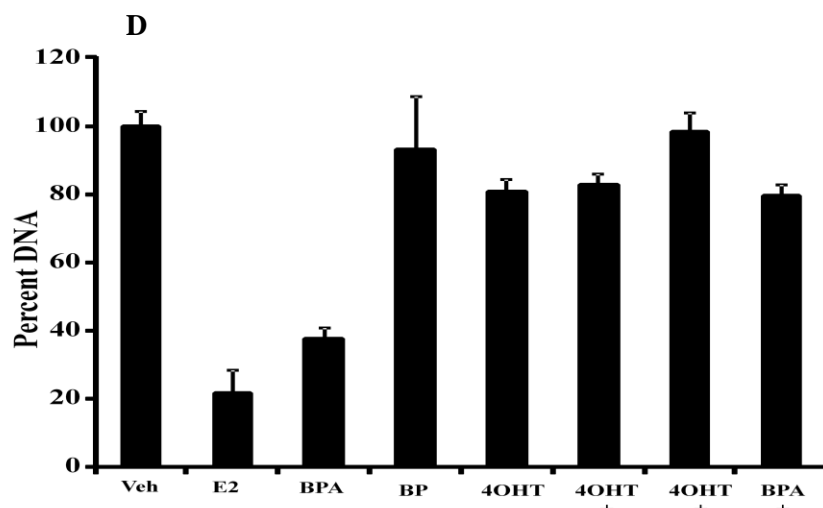


Figure 20. Differential effect of bisphenol (BP) and bisphenol-C (BPA) on growth of MCF7:WS8 cells treated for six days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a six day period. The growth is measured as amount of DNA present in each well. B. Dose dependent effect of BP, BPA and E₂ on apoptosis of MCF7:5C cells treated for six days as indicated. The black bar denotes the level of DNA in vehicle treated cells over a six day period. The growth is measured as amount of DNA present in each well. C. Dose dependent effect of BP and BPA on E₂ (1nM)-induced apoptosis in MCF7:5C cells, treated over a six day period. The growth is measured as amount of DNA present in each well. D. Effect of BP and 4OHT on BPA induced apoptosis in MCF7:5C cells over six day period. The data is presented as percent of growth considering the vehicle treated cells as 100 percent. Each value is average of at least three replicates +/- S.D.

Regulation of estrogen responsive gene trefoil factor 1(TFF1 or PS2) by bisphenol and bisphenol-A

We next investigated the transcriptional regulation of a characterized estrogen-regulated gene, TFF1 (PS2) by BP and BPA and compared it with E₂ and 4OHT. MCF7:WS8 cells were treated for 6 hours with the 0.1% ethanol (veh), E₂ (10⁻⁹M), 4OHT (10⁻⁶M), BP (10⁻⁶M and 10⁻⁵M) or BPA (10⁻⁶M and 10⁻⁵M) and total RNA was harvested using TriZol reagent. Two different concentrations (10⁻⁶M and 10⁻⁵M) were used for BP and BPA because BPA is a weak estrogen and we wanted to evaluate the concentration dependent regulation of these compounds. As expected, PS2 mRNA was up-regulated around five fold by E₂ (10⁻⁹M) compared to vehicle treatment and 4OHT (10⁻⁶M) completely failed to induce the levels of PS2 mRNA (Fig. 21). On the other hand, BP treatment at 10⁻⁶M concentration moderately (~2 fold) up-regulated the PS2 mRNA levels and higher concentration (10⁻⁵M) of BP did not further increase the levels of PS2 (Fig. 21). Conversely, cells treated with BPA exhibited dose dependent increase in up-regulation of the PS2 mRNA and the magnitude of up-regulation with high concentration (10⁻⁵M) of BP was equivalent to the E₂-mediated up-regulation of PS2 mRNA (Fig. 21). These results clearly indicate the differential transcriptional activation of PS2 mRNA as BPA treatment at higher concentration achieved the PS2 activation comparable with E₂, but BP treatment failed to do so.

Figure 22

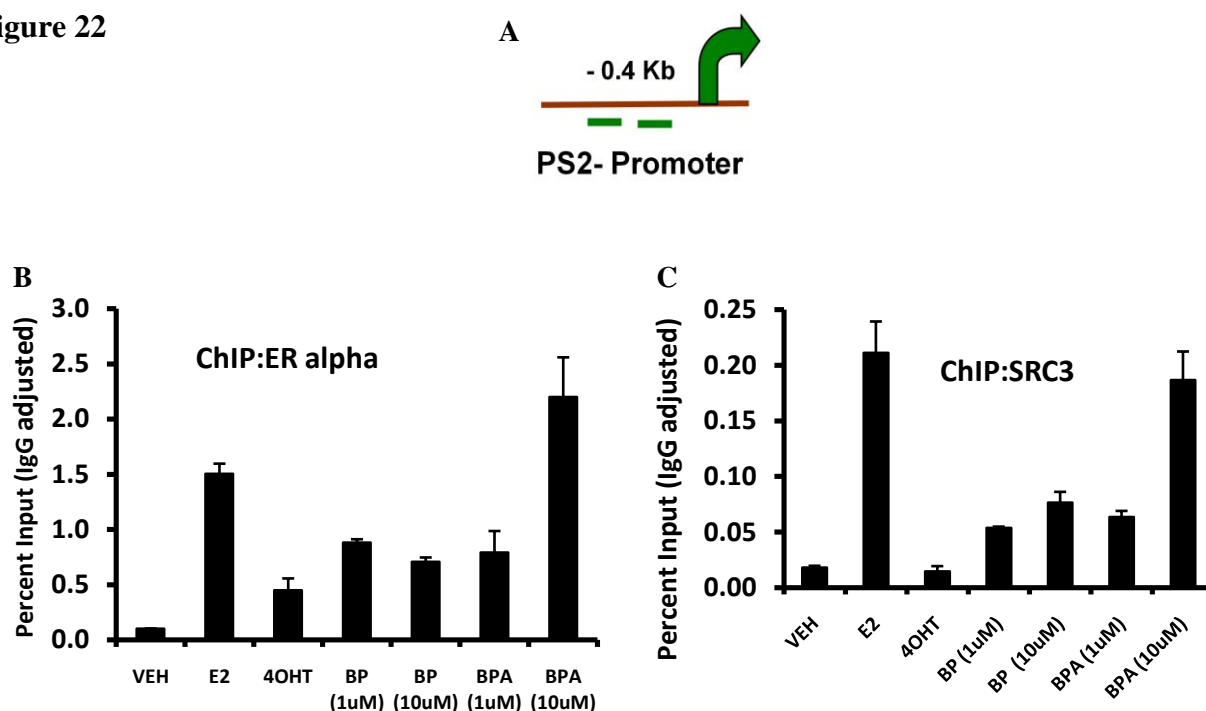


Figure 22. Recruitment of estrogen receptor alpha (ER alpha) and steroid receptor co-activator-3 (SRC3) at the estrogen responsive element (ERE) of proximal promoter of PS2 gene followed by 45 minutes treatments of bisphenol (BP), bisphenol-A (BPA) compared with 17-beta estradiol (E_2) and 4-hydroxy-tamoxifen (4OHT) in MCF7:WS8 cells. **A.** Schematic representation of the PS2 proximal promoter containing an ERE. **B.** Recruitment of ER alpha at the PS2 proximal promoter, by ChIP assay after 45 minutes of indicated treatment. All the values are represented as percent input of the starting chromatin material and after subtracting the IgG control for each sample. **C.** Recruitment of SRC3 at the PS2 proximal promoter, by ChIP assay after 45 minutes of indicated treatment. All the values are represented as percent input of the starting chromatin material and after subtracting the IgG control for each sample.

Differential induction of transformlpi 'i tqy vj 'hcevt'errj c'VI H +i gpg'd{ 'Dkrj gpqilcpf " Bisphenol-A in MDA: MB-453"egm'uædn{ "tcpuhevgf "y kj "y kf "v{ r g"GT "qt "F 573I " o wcpvGT 0

Previous studies from our laboratory have established an *in vitro* system to evaluate and differentiate the conlqto cvkqp"qh"rki cpf gf "GT "kpf wegf "d{ "r rcpct"cpf "ppp-planar ligands. Cevkcvkqp"qh"VI H "i gpg"kp"O F C<"O D"453"egm'uædn{ "tcpuhevgf "y kj "y kf "v{ r g"GT "O E4" egm"qt"o wcpvGT "F 573I +*LO 8"egm"y j lej "j cxg"cp"cur ct cvg"uædukwgf "y kj "i n{ elpg"cv" the 351 co kpq"cekf "r qukkqp."ku"wgf "cu"o ctngt "v{ f kkpki wkuj "y j g"GT "kpvgtcvkvpu"dgvy ggp" planar and non-planar estrogen ligands (25). We treated the MC2 and JM6 cells with increasing eqpegpvcvkvpu"qh"DR"cpf "DRC"cpf "o gcuwtgf "y j g"VI H "kpf wekqp"kp"y gug"egm"O E2 was used as a r qukkxg"eqpvtqrO"kp"O E4"egm."y j lej "j cxg"uædn{ "tcpuhevgf "y kf "v{ r g"y v"GT ".cm"y j g"vguf " rki cpf u"kp wegf "VI H "tcpuetkr v"r xgn"to similar levels (Fig. 23A-0"kp wekqp"qh"VI H "d{ "DRC" was observed at higher concentrations whereas BP and E2 had similar effects (Fig. 23A). On the qvj gt"j cpf ."kp"LO 8"egm."y j lej "ctg"uædn{ "tcpuhevgf "y kj "o wcpv"F 573I +*GT ".DR"y cu"pqv" able to induce VI H "tcpuetkr vqp"gxgp"cv"j ki j gt"eqpegpvcvkvpu"Hi . 23B), whereas E2 and BPA tgcvo gpv"kp wegf "VI H "Hi . 23B), although the maximal induction with BPA was observed at

higher concentration which was less than half of E₂ treatment. We further confirmed that E₂-dependent manner; whereas co-treatment of BPA in presence of E₂ failed to inhibit it (Fig. 23C). These results further confirmed that BPA and E₂ interacted with ER and BPA was a weaker ligand than E₂. Different as it required the aspartate at 351 rqukkqp"qh'GT "r tqvklp"vq"lpf weg"vj g"VI H . "y j lej "tgugo drgu"lpvgtcevqp"qh'GT "y kj "pqp-planar estrogen molecules (25).

Figure 23

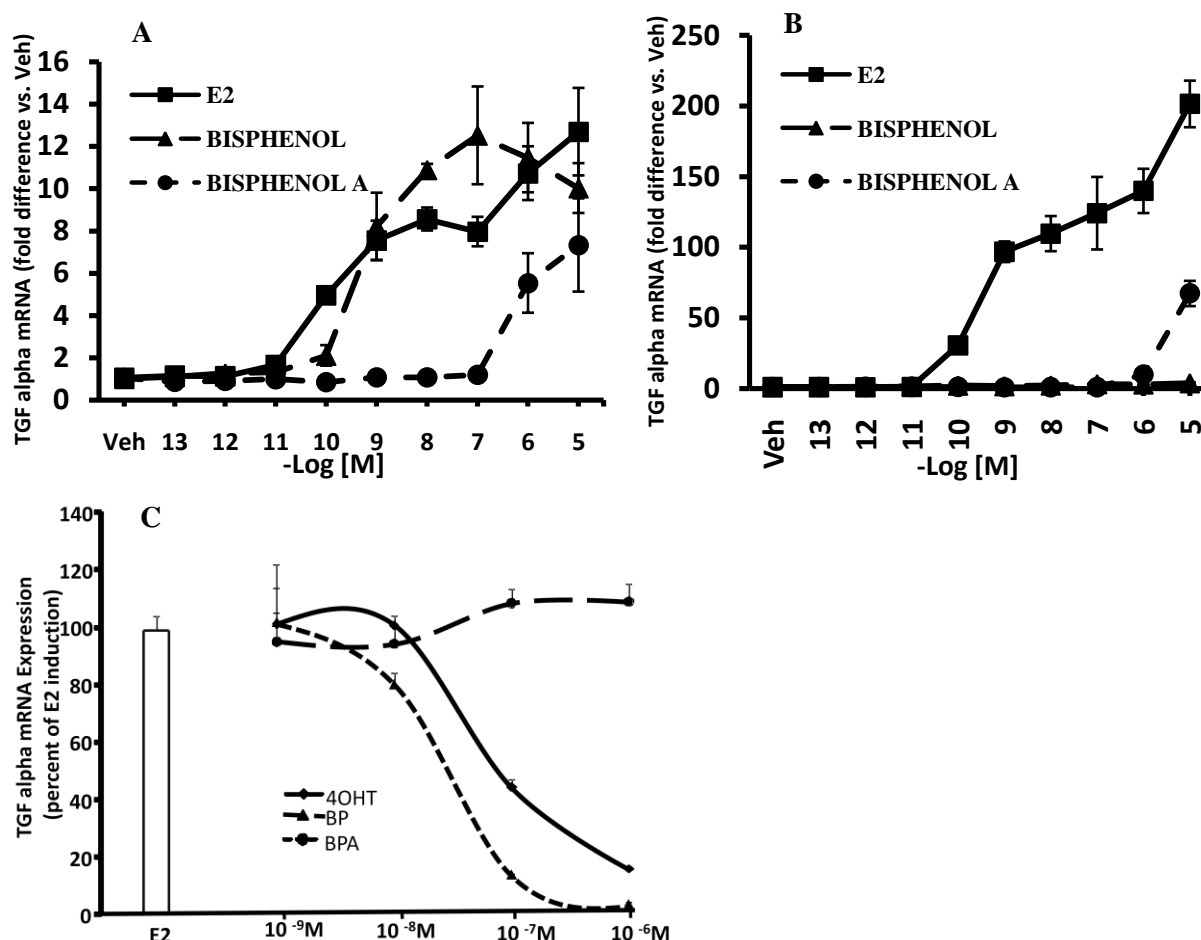


Figure 23. Induction of VI H 'b TPC'd{ 'G₂, BP, and BPA in MDA:MB 231 cells stably transfected with y kf 'Vf rg'GT 'O E4'egmu"qt 'F573I 'b wcpv'GT 'LO 8'egmu"0C0 O E4'egmu"y gtg"v tgcvgf "y kj "39 "gwtcf kqi" (E₂), bisphenol (BP) or bisphenol-A (BPA) at indicated concentration for 48 hrs and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RT-PCR) was performed to assess the expression of VI H "wukpi "58D6"cu"cp"lpvgtpcn'eqptqr0'Vj g"xcnngu"ctg"r tguqpvgf "cu"lqrf "f khtgpgg"xtguu"xgj kerg"v tgcvgf "egmu. B. LO 8'egmu" y gtg"v tgcvgf "y kj "39 "gwtcf kqi" *G₂), bisphenol (BP) or bisphenol-A (BPA) at indicated concentrations for 48 hrs and cells were harvested for total RNA. Total RNA was reverse transcribed and real time PCR (RT-PCR) was performed to assess the gztguukqp"qh'VI H "wukpi "58D6"cu"cp"lpvgtpcn'eqptqr0'Vj g" values are presented as fold difference versus vehicle treated cells. C. JM6 cells were treated with E₂ alone or in combination with different concentration of bisphenol (BP), bisphenol-A (BPA) or 4-hydroxy tamoxifen (4OHT) cu"lpf lecvf "hqt"6: "j tu0Vj g"xcnngu"ctg"r tguqpvgf "cu"r gtegpvc i g"qh'gztguukqp"qh'VI H "o TPC"eqpukf gt lpi "vj g"G₂-induced levels as 100 percent.

O qngewært 'f qenlpi 'qh'DR'c'pf 'DRC'v'j g'NDF 'qh'GT'c'j c''

Vq"fgvto kpg"vj g"dlpf lpi "o qf g"qh'DRC"cpf 'DR'v'GT' . 'vj g"rki cpf u'y gtg"f qengf "v'j g" ci qpkv"cpf "cpvci qpkv"eqphqto cvkpu"qh"vj g"tgegr vqt0'Vj g"gzr gtlo gpvcn'utwewtg."5GTV."y cu" ugrgevgf "h'qo "RF D" hqt"vj g"cpvci qpkv"eqphqto cvkqp"qh" "Hki 0'46C+" y j krg" hqt"vj g"ci qpkv" eqphqto cvkqp" y q"gzr gtlo gpvcn'utwewtg"y gtg"ugrgevgf ."pco gn"vj g"tgegr vqt"eq/et { ucnk gf " y kj "G4."3I Y T"Hki 046D+"cpf 'F GU."5GTF "Hki 046E+"tgr gevkg"0'

C"eqo o qpn" wugf "o gj qf "v'gxcnvcg"vj g"f qenlpi "o gj qf "ghlekgpe { "ku"v'f qenl'vj g"eq/ et { ucnk gf " rki cpf " v'ku"pcvkg"gzr gtlo gpvcn'utwewtg0' Vj g"gzr gevfg "qweqo g"y qwf "dg" c" f qenlpi "uqnwkp."r qug."y j lej "tgecr kwrcvu"vj g"dlpf lpi "o qf g"qh'vj g"rki cpf "lp"vj g"dlpf lpi "ukg"qh" vj g"gzr gtlo gpvcn'utwewtg0' Hqt"vj ku"tgcup."5F/eqphqto cvkpu"qh" G4."F GU"cpf "6QJ V"y gtg" i gpgtcvgf ."qr vko k gf "y kj "O O HH, 6"hqteg"hgfn"cpf "vj gp"uwdlgevfg "v'r tgr ctcvkqp" hqt" f qenlpi " wulpi "vj g" Nki Rtgr "wkrk { 0' Vj g"uco g"r tqvceqn"j cu"dggp" hmqy gf "hqt" DRC" cpf "DR0' Rtqvgk" Rtgr ctcvkqp"Y qtnmqy "Uej t3/4 lpi gt."NNE."P gy "[qtm"P [."4233+"y cu"go r mq { gf "v'r tgr ctg"vj g" r tqvgkpu" hqt"o qngewært "f qenlpi 0'Vj g"tgulf wgu"y gm/npqy p"v'dg"ko r qtcvp" hqt"dkmqi kcn'cevkv { " F 573"cpf "G575"y gtg"ngr v'ej cti gf "lp"cm'vj tgg'tgegr vqtu."vj g"tgg'tqcvkqp"qh" { f tqz { n'i tqwr "hqt" V569"y cu" cmqy gf " cpf " J 746"tgulf wg"y cu" r tqvpcvgf "cv"vj g"gr uknp" pktqi gp" cvqo "lp"vj g" eqo r ngzgu"3I Y T"cpf "5GTV"dcugf "qp"vj g"cxckrdrg"rkgtcwgt"fcv0'Kp"vj g"ecug"qh"5GTF "eqo r ngz" y q"utwewtg"y gtg'r tgr ctgf "hqt" f qenlpi "twpu"j cxlpi "J 746"r tqvpcvgf "cv"gr uknp"5GTF a "+"cpf " f gnc"5GTF a "+"pktqi gp0"

Vj g"dgu" f qenlpi "r qugu"y gtg"ugrgevgf "dcugf "qp"vj g"eqo r qukg"ueqtg."Go qf gn"y j lej " ceeqwpw"pqv'qpn" hqt"vj g"dlpf lpi "chhpkv { "dw'cnq" hqt"vj g"gpgti gve"vto u."uwej "cu"rki cpf "utckp" gpgti { "cpf "lpvgtcevkqp"gpgti { 0'Y j gp"G4."F GU"cpf "6QJ V"y gtg"f qengf "v'j gkt"pcvkg"utwewtg" vj g"vr "tcpngf "f qenlpi "uqnwkp"j cxg" c"rki cpf "TO UF"qh"20575" hqt"G4."20638" hqt" F GU" f qengf "v'j 5GTF a "cpf "20594"y j gp" f qengf "v'j 5GTF a ."cpf "2084; "hqt"6QJ V0"

Vj g"r tgf levfg "dlpf lpi "o qf gu"qh'DR"v'j g"qr gp"cpf "emugf "eqphqto cvkqp"qh"GT"ctg" ulo krt." hqto lpi "vj g"J /dqp" pgvy qtm' dgvy ggp" G575." T5; 6" cpf "vj g"j ki j n { qtf gtgf "y cvgt" o qngewært "cpf "cp"cf f kkpkn"J /dqp" y kj "vj g"j { f tqz { n'i tqwr "qh"V569"Hki 046I ."46J ."46K0'Vj g" eqo r qukg"ueqtg."Go qf gn"uj qy u"vj cv'DR"ku"dgwgt"cee qo o qf cvgf "lp"vj g"dlpf lpi "ukg"qh"vj g"qr gp" qt" cpvci qpkv"eqphqto cvkqp"qh" GT " cpf "k'ku"o qtg" rkngn { " hqt"vj g"rki cpf "v'ku"dlpf "cv"vj ku" eqphqto cvkqp"qh"GT0'Ulo krt"tguwmu"j cxg"dggp"qdvclpgf "wulpi "vj g"kp" wegf "Hk" f qenlpi "o gj qf ." y j lej "cee qwpw" hqt"dqy "vj g"rki cpf "cpf "r tqvgk"rgz kdkv { "56+0'

Kp"ecug"qh'DRC"y q"j ki j n { "r tqdcdrng"dlpf lpi "o qf gu"j cxg"dggp"kf gpvkhgf 0'Vj g"htuv"qpg" j cu"dggp"o quw { "r tgf levfg "y j gp"vj g"rki cpf "j cu"dggp" f qengf "lpv"vj g"dlpf lpi "ukgu"qh"GT "eq/ et { ucnk gf "y kj "G4"cpf "F GU."vj g"utwewtg"5GTF a "wulpi "vj g"UR"o qf g0'Vj g"rki cpf "ku"r nægf " cetquu"vj g"dlpf lpi "ukg"lp" c"ulo krt"qtlgpvcvqp"y kj "vj g"pcvkg"rki cpf u."j cxlpi "vj g"y q"o gj { n' i tqwr u"lpxqrkgf "lp"j { f tqr j qdle"eqpvcwu"y kj "vj g"ukf g"ej ckpu"qh'co kpq"cekf u"Y 5: 5."N5: 6."N747." cpf " N7620' Cnuq." DRC" hqto u" J /dqp" u" y kj "J 746" cpf " G575" Hki 0' 46G+0' Y j gp" f qenlpi " ecrewrcvqp"j cxg"dggp"twp"lp"vj g"ZR"o qf g"qh'I rkf g" c"ugeqpf "cnki po gpv"qh'vj g"vr "cpngf "r qugu" lp"vj g"dlpf lpi "ukg"qh"5GTF a "cpf "5GTF a "j cu"dggp"pqvlegf 0'Vj ku"qtlgpvcvqp"lpxqrkgu"vj g" hqto cvkqp"qh"J /dqp" u"dgvy ggp"vj g"j { f tqz { n'i tqwr u"qh'DRC"cpf "co kpq"cekf u"I 743."G575"cpf "

T5; 6" *Hk 0' 46H0' Cr ctv' hqto " vj g" J /dqp f u" hqto c v k p." vj g" o g vj { n' i t q w u" ctg" k p x q r k g f " k p" j { f t q r j q d l e" e q p x c e u" y k j " c o k p q" c e k f u" N568." H626." c p f " N64: 0 C n q." vj k u" d k p f k p i " o q f g" j c u" d g g p" g p e q w p v g t g f " h q t" 8" q w" q h" 32" r q u g u" t g u w n g f " h q t o " vj g" f q e n k p i " q h" D R C" k p v q" vj g" g z r g t k o g p v c n' u t w e w t g" 3 I Y T 0"

Y j g p" D R C" k u" f q e n g f " v q" vj g" c p v c i q p k u v' e q p h q t o c v k p p." 5 G T V." k' k u" q t k g p v g f " r g t r g p f k e w r c t" y k j " vj g" d k p f k p i " r q e n g v" c p f " k p" vj k u" c r k i p o g p v' k' j c u" vj g" r t q r g p u k v' " v q" h q t o " vj g" J /d q p f " p g v y q t n i' k p x q r k p i " G575." T5; 6" c p f " c" y c v g t" o q n g e w g" *Hk 0' 46 F +0 C f f k k q p c m' . " c" j { f t q i g p" d q p f " y k j " vj g" j { f t q z { n' i t q w r " q h" V569" k u" h q t o g f 0' k p" vj k u" c r k i p o g p v' vj g" d k p f k p i " u k g" k u" r q q t n' " q e e w r k g f " c p f " vj g" j { f t q r j q d l e" e q p x c e u" y k j " vj g" c o k p q" c e k f u" h k p i " vj g" d q v q o " q h" vj g" d k p f k p i " u k g" c t g" o k u k p i 0"

Vj g" e q o r c t c v k x g" c p c n' u k u" q h" vj g" e q o r q u k g" u e q t g" G o q f g n' h q t" vj g" c i q p k u v' c p f " c p v c i q p k u v' v q r " t c p n g f " f q e n k p i " r q u g u" q h" D R C" j c u" u j q y p" vj c v' vj g" d k p f k p i " o q f g" r t g f k e v g f " h q t" vj g" c p v c i q p k u v' e q p h q t o c v k p p" k u" j k i j n' " k o r t q d c d n g" c p f " k' k u" o q t g" r k n g n' " h q t" D R C" v q" d k p f " v q" c" e q p h q t o c v k p p" q h" G T " e m q u n' " t g r v g f " y k j " vj g" c i q p k u v' q p g 0' Q p" vj g" q v j g t" j c p f . " v y q" f k u k p e v' d k p f k p i " o q f g u" q h" D R C" v q" vj g" c i q p k u v' e q p h q t o c v k p p u" q h" G T " j c x g" d g g p" r t g f k e v g f " y k j " v k i j v" G o q f g n' u e q t g u" c p f " e c p p q v' d g" e n g c t n' " f k u e t k o k p c v g f " y j k e j " c r k i p o g p v' k u" e q t t g e v' q t" c v" n g c u v' y k j " vj g" j k i j g u v' r t q d c d k r k v' " q h" d g k p i " t k i j v 0'

Vj g" f q e n k p i " u e q t g u" e c r e w r v g f " h q t" G4." F G U" c p f " D R C" u j q y u" vj g" d k p f k p i " c h h k p k v' " q h" D R C" v q" G T " k u" o w e j " n y g t" y j g p" e q o r c t g f " y k j " vj g" d k p f k p i " c h h k p k g u" q h" G4" q t" F G U" v q" G T " 0' Vj g u g" t g u w n u" c t g" k p" g z e g n g p v' c i t g g o g p v' y k j " d k q n i k e c n' g z r g t k o g p v c n' f c v c 0'

"
"
"
"
"
"
"
"
"
"
"
"

Figure 24

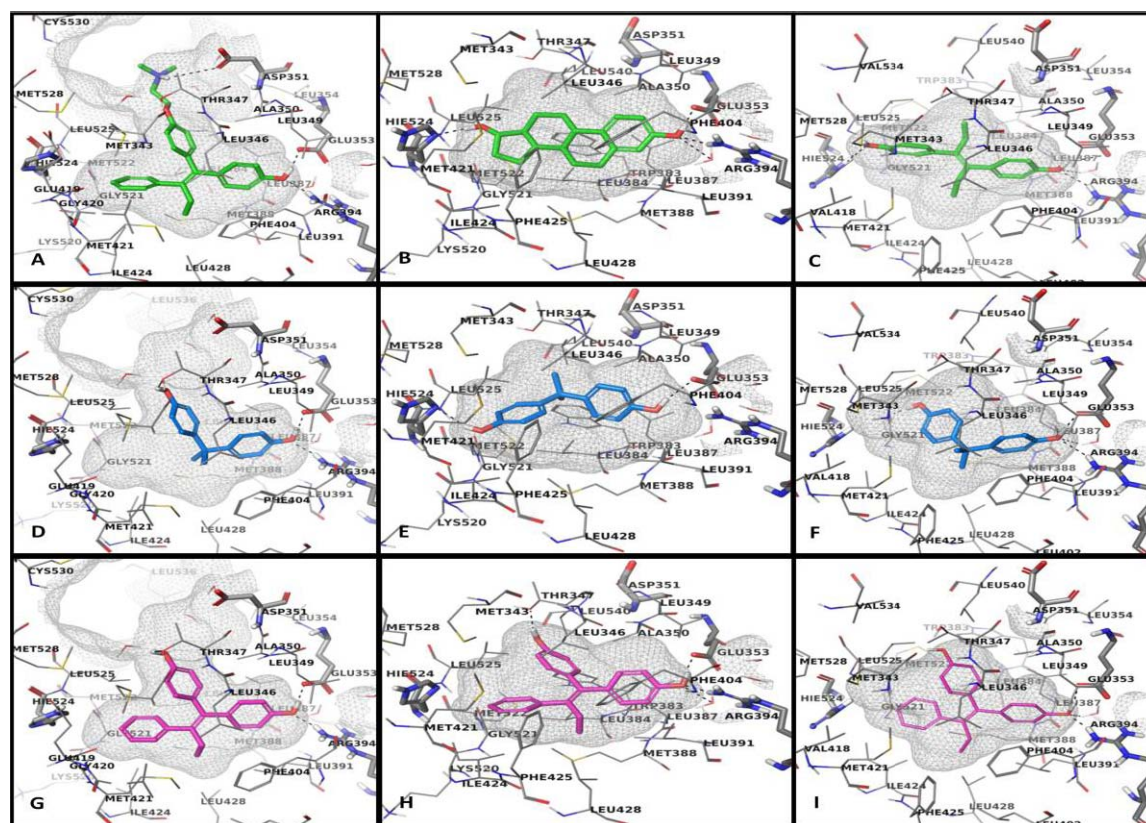


Figure 24. Cross-usage of the top ranked docking poses of BPA into the binding site of 3ERT (D), 1GWR (E), 3ERD (F) are displayed with C atoms colored in magenta while the best docking solutions of BP computed for 3ERT (G), 1GWR (H), 3ERD (I) are represented with C atoms colored in blue. The amino acids involved in H-bond contacts are depicted as sticks and the rest of the amino acids lining the binding site are shown as lines having the C atoms colored in gray. Only polar hydrogen atoms are shown, for simplicity.

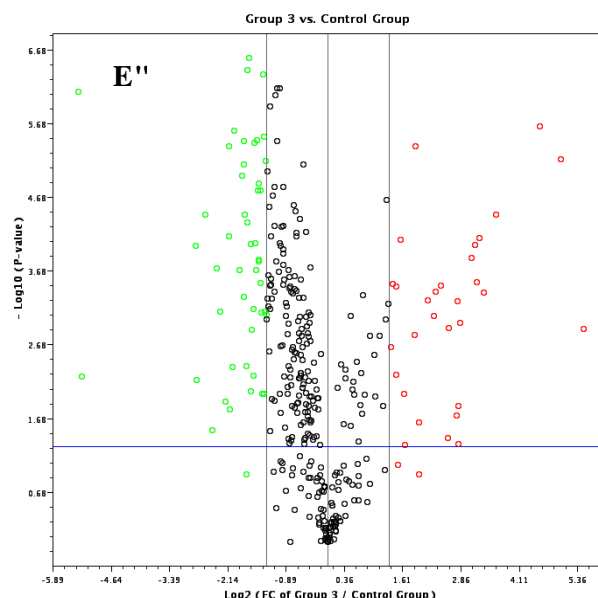
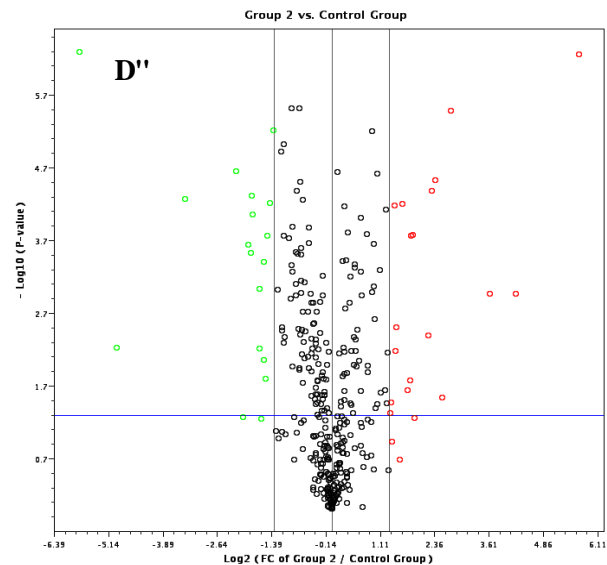
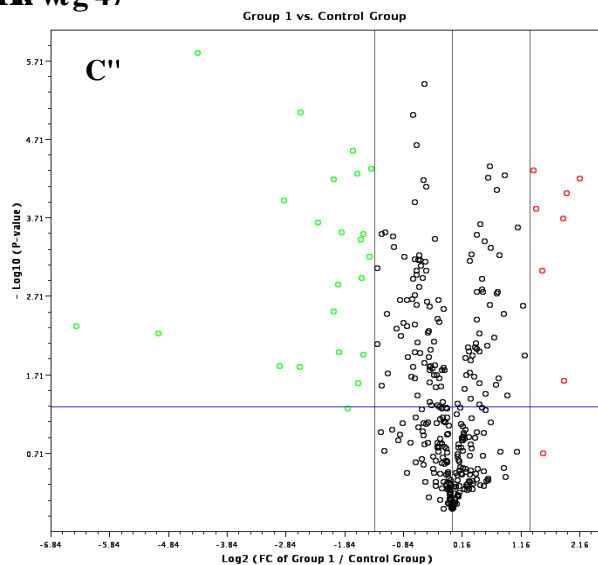
Comparative analysis of regulation of apoptotic genes by BP, BPA, 4OHT and E₂ in MCF7:5C cells using apoptotic gene RT-PCR profiler

We thereafter determined the effect of BP and BPA treatment in regulating the apoptosis related genes in MCF7:5C cells and compared it with E₂ and 4OHT as a positive and negative inducer of apoptosis respectively. We used the RT-PCR profiler assay kits for apoptosis from a commercial vendor which uses 384 well plates to profile the expression of 370 apoptosis related human genes (Qiagen; SABiosciences Corp, Fredrick, MD; Cat#330231 PAHS-3012E). All the treatment with the ligands we first treated the MCF7:5C cells with E₂ (10⁻⁹M) for 24, 48 and 72 hrs (in triplicates) and created an apoptotic gene signature throughout these time points after comparing them with vehicle treatment (Fig. 25A, 25B and 25C and Table 1). This gene

uli pcwtg" y cu" i gpgtcvgf " d{ " eqo r ctłpi " vj g" gztgtuakqp" rŋxgn' qh" cm' vj g" i gpgu" y kj " xgj keng" vtgcvo gpv'cvf "ugrgevpki "vj g'i gpgu" y j lej "y gtg'cv'rgcu'40/" hqrf "qxgt/ gztgtuugf "qt'wpf gt/ gztgtuugf " cu'eqo r ctgf "vq" xgj keng" vtgcvgf "egm0Vj g" hqrf "ej cpi g" y cu'ecrēwrcvgf "d{ " f gnc/ f gnc" E v' o gvj qf " wulpi " vj g" y gd" dcugf " vqpn" TV⁴ " r tqhkrŋ" RET" ctte{ " f cwc" cpcn{uku" xgtukqp" 50/" *S kci gp= UCDkquekgpegu'Eqtr. "Hgf tlem" OF +0"

Chgt"ectghwm{ "cpcn{ lpi "vj g" i gpg" rku' i gpgtcvgf "d{ "G₄" vtgcvo gpv' qxgt" vj g" cdqxs" uckf " vko g'r gtlkf "y g'ugrgevgf "6: "j tu'cu'vj g' vko g'r qkp'v'vq' vtgcvo EH9-7E" egm' y kj "DR" DRC" cpf "6QJ V" cpf "eqo r ctg" vj g" gztgtuakqp" qh' vj g" cr qr vquku' tgrēvgf " i gpgu" y kj " vj g" i gpg" uli pcwtg" qh' vj g" G₄ vtgcvo gpv'cv'6: "j tu0Vj ku' r ct'kewrct" vko g'r qkp'v' y cu'ugrgevgf "dgecwug" vj g" O EH9-7E" egm' wpf gti q" cr qr vqle" ej cpi gu' chgt "G₄" vtgcvo gpv' f wtkpi "vj ku' vko g'r gtlkf " *: + "cpf "cnuq" dgecwug" chgt "6: "j tu' qh' G₄" vtgcvo gpv' vj g" egm' ctg" eqo o kwgf "vq" cr qr vquku. "cu'6QJ V" vtgcvo gpv' ecppqv' tguewg" vj gug' egm' chgt "vj ku' vko g'r qkp'v' r ŋcug" ugg" Vcum'4d/7. "Hi 04; +0"

Hi wt g'47"



Hi wt g'470Tgrt gupwclqp' qh' G₄ "3pO +tgi wrcvgf " cr qr vqle" i gpgu' lp' O EH9-7E" egm' cv'46. "6: "cpf " 94" j tu' qh' vtgcvo gpv' xgtuww' xgj keng" vtgcvo gpv' wulpi "xqrecpq" r rŋv' C. "D" cpf "E" ctg" vj g" xqrecpq" r rŋv' qh' G₄/tgi wrcvgf " cr qr vqle" i gpgu' cv'46" j tu. "6: " j tu' cpf "94" j tu' tgr ge'xgn' 0' Gcej "ekeng" lp" vj g" r rŋv' tgrt gupw" qp g" i gpg0I gpgu" y j lej "ctg" wr /tgi wrcvgf " cv'rgcu'40/" hqrf "qxgt" xgj keng" vtgcvo gpv' ctg" f gpqvgf " cu' tgf "ekergu" y j gtgcu' vj g" i gpgu" y j lej "ctg" f qy p/ tgi wrcvgf "cv'rgcu'40/" hqrf "qxgt" xgj keng" ctg" lp" i tggp" ekergu0Vj g" i gpgu' tgrt gupwvgf "d{ " drcen' ekergu" y gtg" pqv' eqpukf gtgf " cu" f khtgtpwcm{ " tgi wrcvgf 0' Vj g" ekergu' cdqxs" vj g" dŋwg" j qtk' qpvcn' rkp g" tgrt gupv' vj g" i gpgu" y j lej "cej kxgu" vj g" uvc'wkrēcn' uli phtēcpeg" qh' r "xcŋwg" qh'2Œ70"

Vcdrg'3''

24 Hrs E₂ Treatment 48 Hrs E₂ Treatment 72 Hrs E₂ Treatment

UP-REGULATED		DOWN-REGULATED		UP-REGULATED		DOWN-REGULATED		UP-REGULATED		DOWN-REGULATED	
Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation	Gene Symbol	Fold Regulation
ÖÖÖÜ	1.1	ÖÖDF	0.4	ÖBYÖF	1.1	ÖBÖ	0.4	ÖBYÖF	1.1	ÖBÖ F	0.4
ÖÖÜSG	1.1	ÖÖSH	0.4	ÖÖSGFF	1.1	ÖÖD	0.4	ÖZVF	1.1	ÖBVF	0.4
ÖÖFU	0.4	ÖÖ	0.4	ÖÖpÖ	1.1	ÖÖDF	0.4	ÖÖÖH	1.1	ÖBÖDF	0.4
pUÖG	1.1	ÖÖÜJ	0.4	ÖÖÖ	0.4	ÖÖSH	0.4	ÖÖSGF	1.1	ÖÖD	0.4
UT ÖÖUF	1.1	ÖÖ	0.4	ÖÖÜÖ	0.4	ÖÖ	0.4	ÖÖpÖ	1.1	ÖÖDF	0.4
VSUG	0.4	ÖÖÖG	0.4	ÖÖÜF	0.4	ÖÖÖG	0.4	ÖÖÖH	0.4	ÖÖH	0.4
VpÖÜÖGF	0.4	ÖÖG	0.4	ÖÖÖ	1.1	ÖÖG	0.4	ÖÖ	0.4	ÖÖSF	0.4
VUÖ GSF	0.4	ÖÖH	0.4	ÖÖUSF	1.1	ÖÖH	0.4	ÖÖÜF	0.4	ÖÖSGF	0.4
		ÖÖ	0.4	ÖÖUSG	1.1	ÖÖ	0.4	ÖÖ	1.1	ÖÖSH	0.4
		ÖPÖE	0.4	PÖUSG	0.4	SÖS	0.4	ÖÖÖ	1.1	ÖÖ	0.4
		ÖPÖE	0.4	ÖÖF	1.1	T UÖUF	0.4	ÖÜYÖ	1.1	ÖÖS	0.4
		SÖS	0.4	ÖF	0.4	pUY	0.4	ÖÖUSF	0.4	ÖÖÖF	0.4
		T UÖUF	0.4	RT Y	1.1	UÖY	1.1	ÖÖUSG	1.1	ÖÖÖF	0.4
		T YF	0.4	SÖSUF	1.1	UÖUF	0.4	ÖÖH	1.1	ÖÖÜ	0.4
		pUY	0.4	pÖ	1.1	UÖÖH	0.4	PÖÖF	1.1	ÖÖÜ	0.4
		pUÖUF	0.4	UT ÖÖUF	1.1	VÖ UH	0.4	ÖÖ	1.1	ÖPÖG	0.4
		UÖY	0.4	UVP	0.4	VpÖÖF	0.4	ÖÖF	1.1	ÖÖ	0.4
		UÖÜF	0.4	VSUG	0.4	VÖÖG	0.4	ÖF	1.1	ÖPÖG	0.4
		UÖÖH	0.4	VpÖUÖF	0.4			RT Y	0.4	ÖÖ	0.4
		UÖÖH	0.4	VÖ H	1.1			SÖSUF	1.1	ÖÖG	0.4
		VÖ UH	0.4	ZÖS	1.1			SÖE	0.4	ÖYF	0.4
		VpÖÖF	0.4					SÖ	0.4	PÖÖF	0.4
		VÖÖG	0.4					T ÖS	0.4	PT ÖF	0.4
								pÖ	1.1	SÖS	0.4
								pUÖUF	1.1	T ÖÖ	0.4
								UT ÖÖUF	0.4	T ÖUSF	0.4
								VSUG	1.1	T ÖUS ÖG	0.4
								VpÖ	1.1	T UÖUF	0.4
								VpÖÖH	1.1	T VÖF	0.4
								VpÖUÖGF	0.4	T YÖG	0.4
								VÖ H	1.1	PT ÖH	0.4
								ZÖS	1.1	PÖH	0.4
										pUY	0.4
										UÖY	0.4
										ÖSHUG	0.4
										ÖSÖPÖF	0.4
										ÖÖYÖG	0.4
										ÖÖÖF	0.4
										ÖÖS	0.4
										ÖÖUF	0.4
										ÖÖSF	0.4
										ÖÖÖH	0.4
										VÖ UH	0.4
										VÖÖ	0.4
										VÖÖG	0.4
										VÖÖG	0.4
										VÖÖ	0.4
										XÖ	0.4
										ZpÖ H	0.4

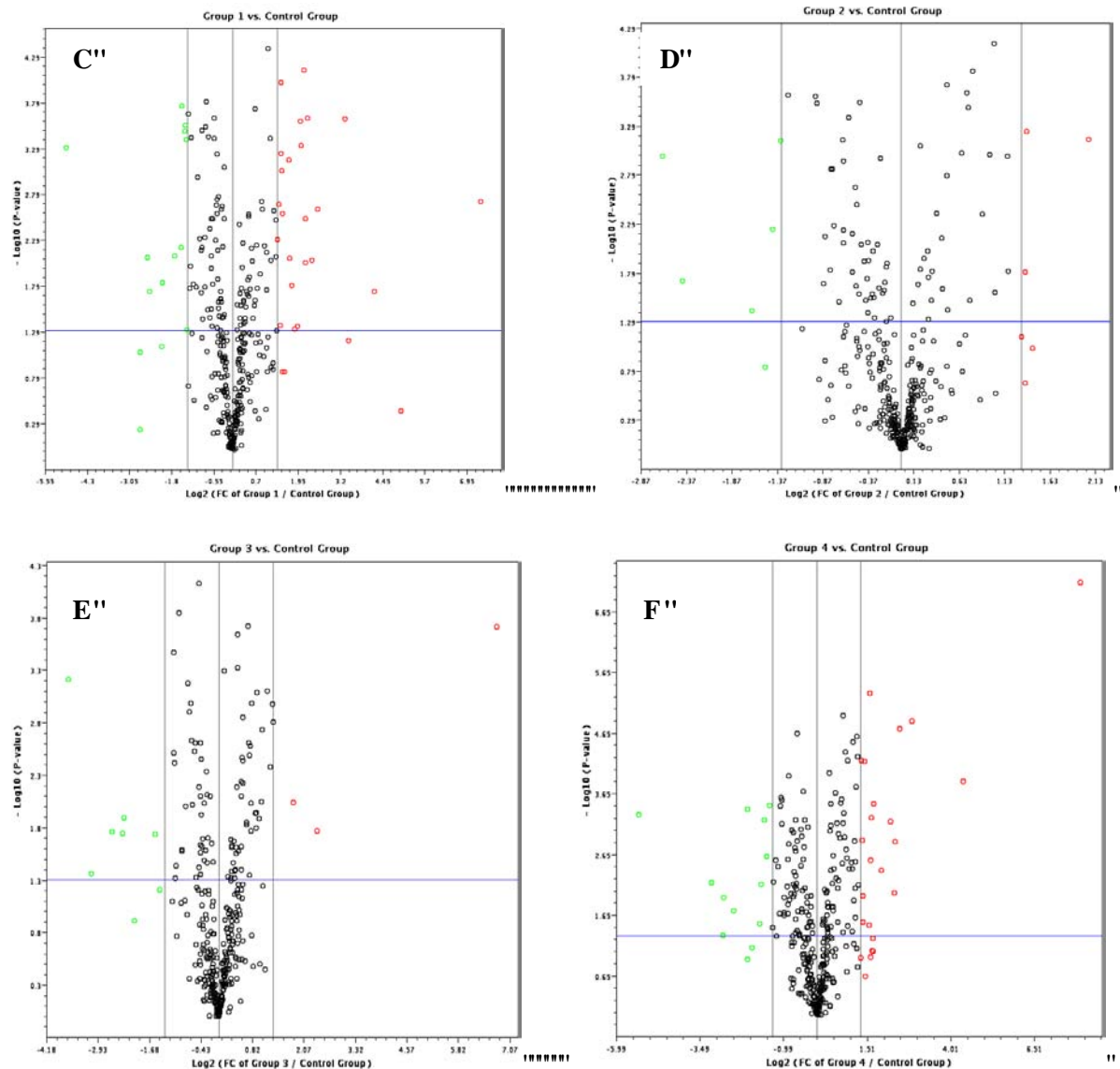
Vcdrg'30I gpg'hk'q'G'3pO +tgi wrcvf 'cr qr vqle'i gpgu'p'O EH9-7E'egm'cv'46.'6: 'cpf '94'j tu'q'htgcwo gpv' xgtuwx'lgj kerg'v'gcwo gpv'O EH9-7E'egm'y gtg'v'gcvgf 'y kj 'xgj kerg'qt'3pO 'G'ht'46.'6: . 'cpf '94'j tu'Vqwn'TP C' y cu'kqrcvgf 'cpf 'tgxgtug'v'cpuetkdgf O'Uwdugs wgpv' 'tgc'v'ko g'RET'y cu'r gthqto gf 'wukpi 'TV/r tqh'krgt'cuuc' 'nku'ht' cr qr vqku'cpf 'y j' i gpgu' y j lej 'y gtg'cv'ngcu' 40' 'hqr' 'w /tgi wrcvgf 'qt' f qy p/tgi wrcvgf 'cu'eqo r ctgf 'v' xgj kerg' v'gcwo gpv'y gtg'ugrvgf 'ht'etgc'v'p' 'y ku' i gpg'rk'v' C'm'y g'v'gcwo gpv'y gtg'r gthqto gf 'p'v'kr decvg.'cpf 'y g'f cvc'ku' r tgu'p'v'f 'cu'cxgtci g'hqr' 'tgi wrcv'p'

P gzv'y g'cpcn' gf 'y g'ej cpi gu'lp'y g'qxgtcm'gzr tgu'kqp'r tqh'krgu'q'ht'cr qr vqle'i gpgu'd' { 'G' . 6QJ V. "DR. "cpf "DRC" xgtuwx'xgj kerg"*Xgj +v'gcwo gpv'cv'6: "j tu"*Hki O'48C. "48D. "48E"cpf "48F " tgr ge'v'xgn' +wukpi 'y g'uco g'cr qr vqku'TV'r tqh'krgt'0"

Hqt"cp{ "i gpg"v"dg"eqpukf gtgf "cu'f khtgt'p'v'cm'f "gzr tgu'ugf 'y g'ugv'y g'ew'q'ht'cu'40' 'hqr' " w' /qt'f qy p/tgi wrcv'p'xgtuwx'y g'xgj kerg'v'gcwo gpv'O'Wukpi 'y ku'etkgt'kqp'y g'etgcvgf "c"i gpg'rkuv' hqt'w' /tgi wrcvgf 'cpf 'f qy p/tgi wrcvgf 'i gpu'ht'gcej 'v'gcwo gpv'i tqw' "Vcdrg'4+0'

"

Hl wt g'48''



Hl wt g'480Tgr t gupwvqp'qh39/ 'gwt cf kpl32/ 'O *G₄+ 'b/j { f tqz{ 'wco qz khp. '32/8' O "6QJ V+ 'dlkr j gpqn'32/8''
O "DR+ 't pf 'dlkr j gpqnC. '32/8' O "DRC+ t gi wv wgf 'cr qr vqle' i gpgu'lp' O EHD<7E 'egm' chgt'6: 'j tu' qh' t gco gpv'
xgtuw'xgj keng'wulpi 'xqrecpq' t nqu' C. 'D. 'E' cpf 'F' 'ctg' y' g' xqrecpq' r nqu' qh' cr qr vqle' i gpgu' t gi wv wgf "d{ 'G₄. "6QJ V. "
DR' cpf "DRC. "t gur gev xgn' "chgt'6: 'j tu' t gco gpv' Gce' "ekerg" k' y' g' r nqv' tgr t gupwv' qpg' i gpg' OI gpgu' y' j kej "ctg" wr /
tgi wv wgf "cv' ngcu' 40' "hqr' "qxgt" xgj keng' t gco gpv' ctg' f gpgw'f "cu' tgf "ekergu' y' j gtgc' u' y' g' i gpgu' y' j kej "ctg' f qy p/
tgi wv wgf "cv' ngcu' 40' "hqr' "qxgt" xgj keng' ctg' k' i tggp' "ekergu' Vj g' i gpgu' tgr t gupw'f "d{ "drcn' ekergu' y' gtg' pqv'
eqpuf gt gf "cu' f kht g'p' km' "tgi wv wgf O' Vj g' "ekergu' cdqxg' y' g' dng' j qtk qpvcn' kpg' tgr t gupw' y' g' i gpgu' y' j kej "cej lxxgu'
y' g' ucw' k' ecn' lki p' k' ecpeg' qh' r' "xcnw' qh' 2070

"

"

"

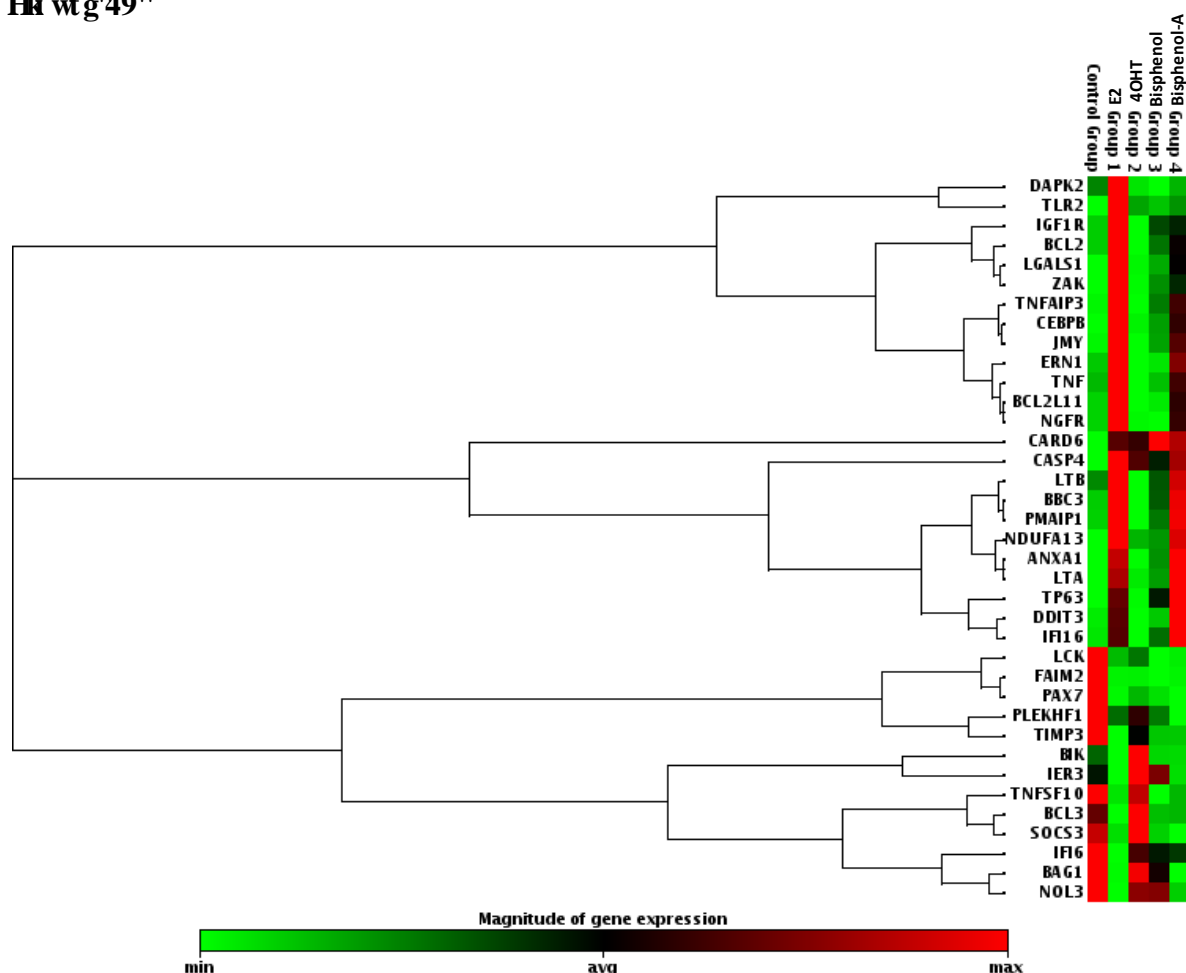
11

11

!!

Y g"vj gtgchgt"i gpgtcvgf"e"j gcv'o cr "Hki 049+'wulpi "vj g"qprkpg"qqn"TV"r tqhkg"RET"cttc{" f cvc"cpcl{uku"xgtukqp"50/"S kci gp=UCDkquekgpegu"Eqtr."Ht gf tlem"O F +lp"y j lej "y g"ugrgevfg"cm" vj g"i gpgu"y j lej "y gtg"cv'ngcu"40/"hqr"wr/"qt"f qy p/tgi wrwvfg"d{"G4"vtgcvo gpv'cpf"eqo r ctgf"kv" y kj "qvj gt"rki cpf"vtgcvo gpv'0Vj ku"j gcv'o cr "engctn{"f go qpwtcvgu"vj cv"vj g"i gpgu"y j lej "ctg"wr / tgi wrwvfg"cv'ngcu"40/"hqr"chgt"6: "j tu"qh"G4"vtgcvo gpv'ctg"pqv"wr/tgi wrwvfg"lp"6QJ V"qt"DR" vtgcvo gpv'0"

Hki wt g'49"



Hki wt g'490J gcv'o cr "qh'crqr wqle"i gpgu"y j lej "ctg"cv'ngcu"40/"hqr"wr/"qt"f qy p/tgi wrwvfg"d{"6: "j tu"qh" vtgcvo gpv'qh39/ 'gwt cf kpi32/"O "G4+'xgt uwxg'keng'cpf"ku't gvwkg'eqo r ct kqp'qh'vj ght 'bzr t gukqp'y kj '6/ j {f tqz{ 'veo qzhgp.'32/"O "6QJ V+'dkur j gpqn'32/"O "DR+cpf'dkur j gpqn'32/"O "DRC+vtgcvo gpv'chgt" 6: "j tu'lp"O EH07E"egm0Vj g"o czko wo "gzr tguugf"ngxgn'qh'cp{"i kxgp"i gpg"ku'tgr tguugvfg"d{"tgf"eqmrt"cpf" o kpko wo "ngxgn'ctg"r tguugvfg"cu"i tggp"eqmrt0Eqptqn'i tqwr"cpf"i tqwr"3."4."5."6"ctg"vj g"tg/r tguugvfg"qh'vj g" xgj keng."G4."6QJ V."DR"cpf"DRC"vtgcvo gpv'tgur gevwgn'0Vj g"i gpg"gzr tguukqp"ngxgn'lp"gej"vtgcvo gpv'i tqwr"ku'vj g" cxgtci g"qh'vj tgg'lpf gr gpf gpv'dkqmi kcn'tgr ncevuo0"

J qy gxgt."o clqtkv{"qh'vj g"i gpgu"wr/tgi wrwvfg"d{"DRC"vtgcvo gpv'ctg"vj g"uco g"i gpgu"wr / tgi wrwvfg"d{"vj g"G4"vtgcvo gpv'0O cp{"qh'vj gug"i gpgu'ctg"wr/tgi wrwvfg"d{"DRC"vq"vj g"uko kct"gz vgpv" cu"G4"cpf"qvj gtu'uj qy "c"fkwpv'vtgpf"qh'qxgt/gzr tguukqp"cu'eqo r ctgf"vq"xgj keng"Hki 049+0"

Vj ku" engctn{ " f knkpi wkuj gu" dgwy ggp" DRC" tgcvo gpv" xgtuwu" DR" tgcvo gpv" cpf " 6QJ V" tgcvo gpv"kp"O EHŒŒE"egmu."cu"DRC"tgcvo gpv'tgugo drgu"emugn{ "vq"G4"tgcvo gpv'y j gtgcu"DR" tgcvo gpv"ku"o qtg"rkng"6QJ V"tgcvo gpv."tgi ctflpi "vj g"wr /tgi wrwgf "i gpgu."guvdrkuj lpi "vj g"hev" vj cv"vj g"o qf g"qh"cevqp"qh"DR"kp"O EHŒŒE"egmu"ku"rkng"cp"ögutqi gp/cpwi qpkuö"cu"kv"hcnu"vq" kpf weg" gztguukqp" qh" i gpgu" kpxqrkgf " kp" cr qr vuku." y kj kp" 6: " j tu" qh" tgcvo gpv" *Hki 0' 49+0' Eqpxgtugn{."DRC"tgcvo gpv'kpf weg" vj g"cr qr vuke"i gpgu"kp"O EHŒŒE"egmu"kp"cu"uko krt"o cppgt"cu" G4"tgcvo gpv"*Hki 0'49+0'Cnuq"d{ "eqo r ctlpi "vj g"i gpg"rkuv"*Vcdrg"4+."y j kej "kpenwf gu"cm'vj g"i gpgu" wr/"qt"f qy p/tgi wrwgf "cv'rgcu'40' "hqr" d{ "vj g"tgcvo gpv."kv"ku"gxkf gpv'vj cv'88" "qh"wr /tgi wrwgf " i gpgu"ctg"eqo o qp"dgy ggp"G4"cpf "DRC"tgcvo gpv"y j gtgcu"kp"eqo r ctluqp"qp{ ": " i gpgu"ctg" eqo o qp{ "wr /tgi wrwgf "d{ "DR"qt"6QJ V"tgcvo gpv0'

Cpqvj gt" kpvgtgukpi " qdugtxcvqp" ku" vj g"hev" vj cv" vj g" f qy p/tgi wrwgf " i gpgu" hqmvy " c" f khtgtpv"rcwgtp0'Vj g"i gpgu" f qy p/tgi wrwgf "d{ "DR"tgcvo gpv'tgugo drg"o qtg"vq"G4"cpf "DRC" tgcvo gpv" vj cp"6QJ V"tgcvo gpv" *Hki 0'49" cpf "Vcdrg"4+."cu"ctqwpf"75" "cpf"83" "qh" f qy p/ tgi wrwgf "i gpgu"d{ "vj g"tgcvo gpv"qh"DR"cpf "DRC"tgur gevkgu{."ctg"eqo o qp"y kj "G4"tgcvo gpv" *Vcdrg"4+0'Vj ku"uwi i guu"vj cv"vj g"eqphqto cvkqpcn'tgs wktgo gpv'qh'rkf cpf gf/"GT "ku" f khtgtpv"ht" wr /tgi wrwgf " cpf " f qy p/tgi wrwgf " qh" i gpgu0' Hwtj gto qtg." kv" kpf kevgu" vj cv" vj g" wr /tgi wrwgf " cr qr vuke" i gpgu" ctg" tgur qpukdrg" ht" kpf velpi " vj g" cr qr vuku" ukpeg" wr /tgi wrwgf " i gpgu" ctg" f khtgtpv"cm{ "tgi wrwgf "d{ "DR"cpf "DRC"dw'pqv'vj g' f qy p/tgi wrwgf "i gpgu0'

Qxgtcm"kp"vj ku"ugevqp"y g"j cxg"guvdrkuj gf "vj cv'vj g"eqphqto cvkqpcn'tgs wktgo gpv'qh'GT / rki cpf gf "eqo r ngz"ku" f khtgtpv"ht"vj g"i tqy vj "cpf"cr qr vuku."d{ "wukpi "vy q"utwewtcm{ "uko krt" šgutqi gple' lii cpf u."dkur j gpqn'cpf "dkur j gpqn/C0Vj g"eqphqto cvkqp"qh'vj g'GT /rki cpf gf "eqo r ngz" pqv'qp{ "f gr gpv"u"wr qp"vj g"ej go kecn'utwewtg"qh'vj g"rki cpf "dw'cnuq"vj g"dkpf lpi "o qf g"qh'vj g" rki cpf "vq"vj g'GT "r tqvklp0'Uwdugs wgpv{."vj g"utwewtcm'eqphqto cvkqp"qh'vj g"eqo r ngz" f kevgu"vj g" kpvgtcevqp" y kj " vj g" eq/cevxcvqtu" qt" eq/tgr tguuqtu" y j kej " ku" gxgpwcm{ "tgur qpukdrg" ht" vj g" vcpuetkr vkqpcn'tgi wrwgf "qh'vj g'i gpgu"cpf "vj g"qxgtcm'hwpevkqpcn'qweqo g0"

"

"

VCUM'4<'*I WLqtf cp+/'Vq'gnwelf cvg'vj g'b qngewrt'b gei cpluo 'qh'G4'kpf weg' 'twt xkxcl'cpf " cr qr vuku' kp" dtgcuv' ecpegt " egmi' t gukxcpv' vq" gkxj gt " UGTO u' qt " mpi /vgt o " gutqi gp" f gr t kcvkqp0'

Vcum'4d/7<'Qdkqtcej "cpf "Lqtf cp"ó"Vq'gnwelf cvg'vj g'etk'lecn'tki i gt "r qlpv'ht" 'gutcf kni' kpf weg' "cr qr vuku'cpf "gztmtg" f khtgtpv'cn'i gpg"gzrtguukqp"kp"eqo r ctluqp"vq"e{ vqvqzle" ej go qvj gter{ 'kpf weg' 'cr qr vuku0'

"

Vcum'4d/7<'Qdkqtcej "cpf "Lqtf cp+/'Uwf kgu"ecttkgf "qw"d{ "F t0'Kg{ kpy c"Qdkqtcej "kp"vj g"Lqtf cp" rcdqtcvqt{ "cv'I gqti gxqy p"Wpkxgtuks{0'

"

Fvgto kpcvqp"qh'vj g'hev'v{ "r qlpv'ht" 'gutcf kni'kpf weg' "cr qr vuku'cpf "eqpvtcu' y kj " e{ vqvqzle'ej go qvj gter{ 'kpf weg' 'cr qr vuku'

"

Kpvt qf wevkqp<'

Gutcf kqri' *G₄+ 'ku' c' ng{ "i tqy vj " unko wwu' hqt " gutqi gp" tgegr vqt " r quklxg" dtgcu' ecpegt0
 Enkplecn'uwf lgu'uw r qtv'yj g'wug'qh'gutqi gpu'lp'yj g'tgcv gpv'qh'gutqi gp'tgegr vqt "*GT+'r quklxg"
 r quwo gpqr cwuci'dtgcu'ecpegt "*57+0U{p vj gvk" j k j "f qug'gutqi gpu'lpf wegf "tgi tguakqp'qh'wo qtu"
 lp' r quwo gpqr cwuci' y qo gp" y kj " cf xcpegf " dtgcu' ecpegt" lp' vj g' hku' gxgt" tgr qtvgf " ecpegt"
 ej go qvj gter { "o gf kvgf "enkplecn'uwf {0Vj ku'y cu'uw r qtvgf "d { "Nqppkpi "cpf "eqngci wgu"*58+'y j q"
 hqwpf " vj cv'7o i "v0f "f kvj {nrkdgutqn'lpf wegf " r ctvkn'vq"eqo r rvgv'tgur qpug"lp"32"qw'qh'56"
 r quwo gpqr cwuci'dtgcu'ecpegt" r cvkpw" y j q"j cf " r tgxkwu" gzj cwukxg"cpvj qto qpg"vj gter {0'C"
 tgegpv'enkplecn'tkcn'*5+'uj qy gf "vj cv'y qo gp"y kj "cf xcpegf "dtgcu'ecpegt"cpf "ces vktgf "tgukwpeg"
 vq'ctqo cvug'lpj kdkqtu."tgur qpfgf "vq"nqy "f qug'gutqi gp0Vj g'wug'qh'eqplwi cvgf "gs wkp"gutqi gpu"
 lp"j { uvgteqo kugf "r quwo gpqr cwuci' y qo gp"tgxgcu'r tqgwkqp"ltqo "vj g"lpkf gpeg"qh'lpxcukxg"
 dtgcu' ecpegt" *59+0 Ncdqtcvqt { " uwf lgu" uj qy " vj cv' r j { ukqni ke" gutqi gp" ecwug" npi " vgt "o
 qz hgp'tgukwcpv'wo qtu'vq'tgi tguu"*5: +0Rtgxkwu'uwf lgu'lp"qwt'nd'j cxg"f go qpwtcvgf "vj cv'G₄"
 lpf wegu'cr qr vuku"lp"npi "vgt "gutqi gp"f gr tkxgf "OEH9"egm)*. +0"I kxgp"vj cv'vj gug'ndqtcvqt { "
 qdugtxcvku'vcpurvg'vq'enkplecn'tgugctej . 'k'ku'ko r gtcvkxg'vq'lpkguki cvg'vj g'o qngewrt'gxgpv'vj cv'
 r tgegf g" vj g" lpf wekqp"qh'cr qr vuku" d { " G₄0" " Ecpegt" ej go qvj gter { " lpf wegu" tcr kf " f gcvj " qh'
 pgqr mwk"egm."dw'gutqi gp"lpf wegf "cr qr vuku"ku"cf gr { gf "gxgp0Y g'j cxg'tgegpv"lf gpvktgf "
 vj g" ugs wgpvkn" o gej cpluo " vj cv' rgu" dgj lpf " G₄ " lpf wegf " cr qr vuku" wupi " npi " vgt " gutqi gp"
 f gr tkxgf "gutqi gp'tgur qpukxg'dtgcu'ecpegt"egm)*, +"J qy gxgt"xgt { "rkwg'ku'npqy p"qp"vj g"ghgeev"
 qh'e { vqvzke"ej go qvj gter { "lp"vj gug"egm0'Rcenkczgn'ku"o kvke"ur lpf rg"lpj kdkqt"vj cv'r tgxgpw"
 f guvdkrk cvkqp"qh'o letqwdwgu0Vj gug'vzcpgu'ctg"wgf "gz vpukgu"lp"vj g'cf lwxcpv'vj gter { "qh"
 gctn { "dtgcu'ecpegt"cpf "o c { " r qvkvcm" "f getgcug'tkni'qh'ecpegt"tgewtgpeg"cpf "o qtvkn { " *5; +0"
 J gtg"y g"j cxg"f gvgto kpgf "vj g'gutqi gp"etkplecn'tki i gt"r qlpv'hqt"vj g"lpf wekqp"qh'cr qr vuku"cpf "
 gwef cvgf "vj g"f khtgpkvkn'i gpg"gzr tguakqp"cv'vj ku'r qlpv"lp"eqo r ctukqp"vq"r cenkczgn'lpf wegf "
 cr qr vuku"wupi "vj g'uco g'egm'o qf gnu0

Y qtm'Ceego r nkuj gf <'

I t qy vj 'gh'geu'q'h'gust cf kqn'6/j { f t qz { wco qz khp '*6QJ V+čpf 'r'cerksczgnlp'O EHD<7E'egmu'

Nqpi "vgtō "gwtqi gp" f gr tkgf "OEH9"egmī"i tqy "kpf gr gpf gpv" "qh" gwtqi gpu" cpf "kpf weg" cr qr vqukū"lp" yj g'r tgupeg"qh" G4O'Vq' k'f gpvkh' "lpj kdkqt" { "ghgew"qh'r cerkcz gnlp" OEH9>7E"egmī" cpf "gzt rgtg"ku'r qvgpvcn'vq" kpf weg" cr qr vqukū"lp" eqo r ctkuqp"vq" G4. "yj g"egmī"y gtg"uggf gf "lp"46"y gm' r nvgu"cpf "tgcvgf" y kj "f khtg pveqpegpvtc vqpu"qh' yj g"eqo r qwpf u'qxgt" yj g" kpf kcvgf "f c { u'cpf" yj g" F P C"y cu"o gcwtgf "lp" gcej "y gm' wulpi "c" hwtg uegpv" f { g0'Hi wtg"4: C"uj qy u'y j cv" G4" tgcvo gpv' kpf wegu" cr qr vqukū"qh" OEH9>7E"egmī"lp" c" eqpegpvtc vqpf "f gr gpf gpv"o cpgt "cv" yj g" gpf "qh" ulz "f c { u' tgcvo gpv" kpf "eqpvtcu" yj g" egmī" tgo ckp" wptgur qpukxg" vq" cpv gwtqi gp. "6QJ V" dw"tcy gt" 6QJ V" dnqemū" G4"o gf kcvgf "cr qr vqukū" *Hi 0'4: D+0'Rcerkcz gnlpf wegf "tcr k'f "lpj kdkkqp"qh'i tqy yj "lp" c" f qug" f gr gpf gpv" o cpgt "y kj "o czko wo "lpj kdkkqp" cv" 32wO 0' Hkn { "r gtegpv" i tqy yj "lpj kdkkqp" y cu' cej kxgf "d { "46j tu" *Hi 0'4: E+ "y j kej "lpetgcugf "vq" cm quv" 322' "chgt" 6: j tu"qh' tgcvo gpv' *Hi 0' 4: F +0' kpf "eqo r ctkuqp. "G4" f k'f "pqv's wcpvkc vkn { "kpf weg" cr qr vqukū" wvkn' chgt" 94j tu" *Hi 0'4: G' cpf " 4: H+0' Vcmep "vqi gyj gt. "yj ku'uwf { "f go qpwtcvgf "yj cv' yj gug" dtgcuv' ecepgt "egmī" tgur qpf gf "tcr k'f n { "vq"

the death effects of paclitaxel in comparison to E₂ which highlights the diversity of responses that may be related to differential gene expression.

Figure 28

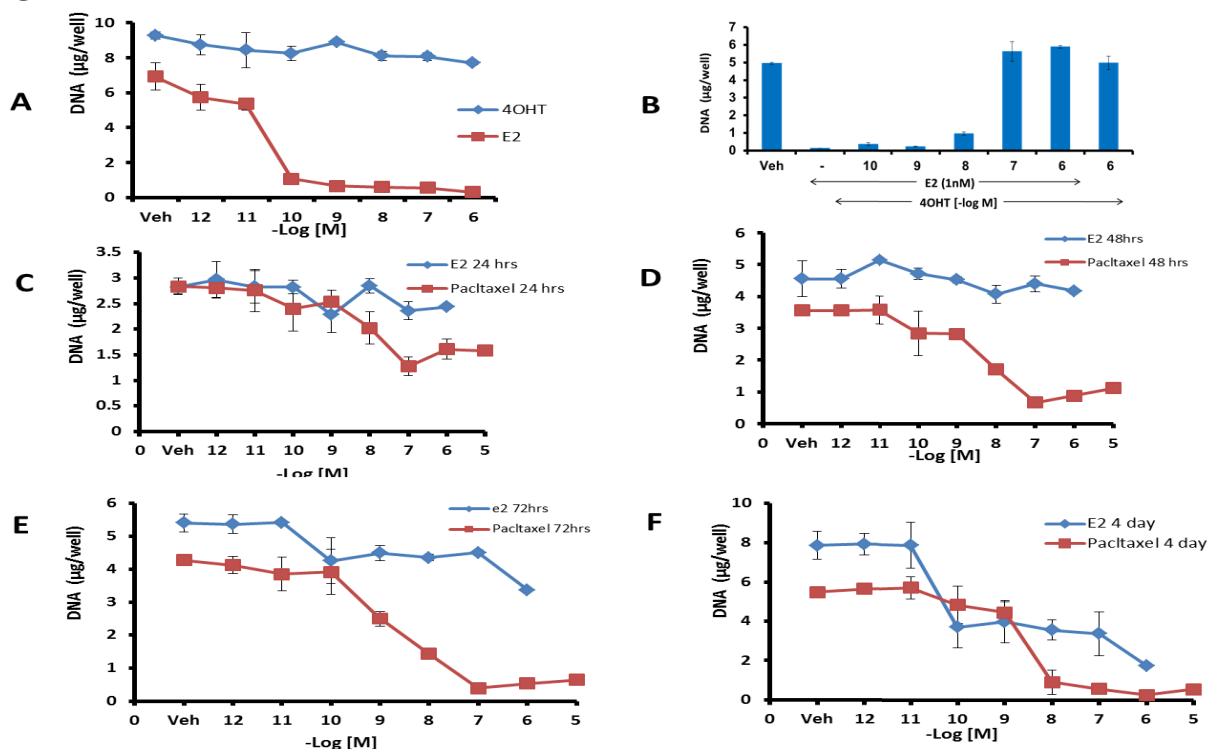


Figure 28. Effect of E₂ and Paclitaxel on the growth characteristics and apoptosis in the MCF7:5C cells. **A.** MCF7:5C cells were seeded in 24-well plate and treated with indicated compounds over a range of doses for six days. Cell growth was assessed as DNA content in each well. **B.** MCF7:5C cells were seeded as in A and cells were treated with either E₂ (1nM) alone or in combination with 1uM 4OHT. MCF7:5C were seeded in 24 well plates and treated with increasing concentrations of paclitaxel and E2 and cells were harvested after 24hrs (**C**), 48hrs (**D**), 72 hrs (**E**) and 96hrs (**F**). The extent of apoptosis was determined by measuring the DNA content of the remaining cells in each well. Each data point shown is average of 3 replicate +/- SD.

Determination of the critical trigger point of estradiol induced apoptosis

To further investigate the delayed response to E₂ mediated apoptosis, MCF7:5C cells were treated with 1 nM of E₂ and subsequently 1uM of 4OHT was used to block the apoptotic effects of E₂ at the indicated time points. 4OHT was able to reverse E₂ mediated apoptosis up to 24hrs, where thereafter, it lost the ability to block apoptosis (Fig. 29). This indicates that the critical trigger point for induction of apoptosis by E₂ lies between 24hrs and 48hrs.

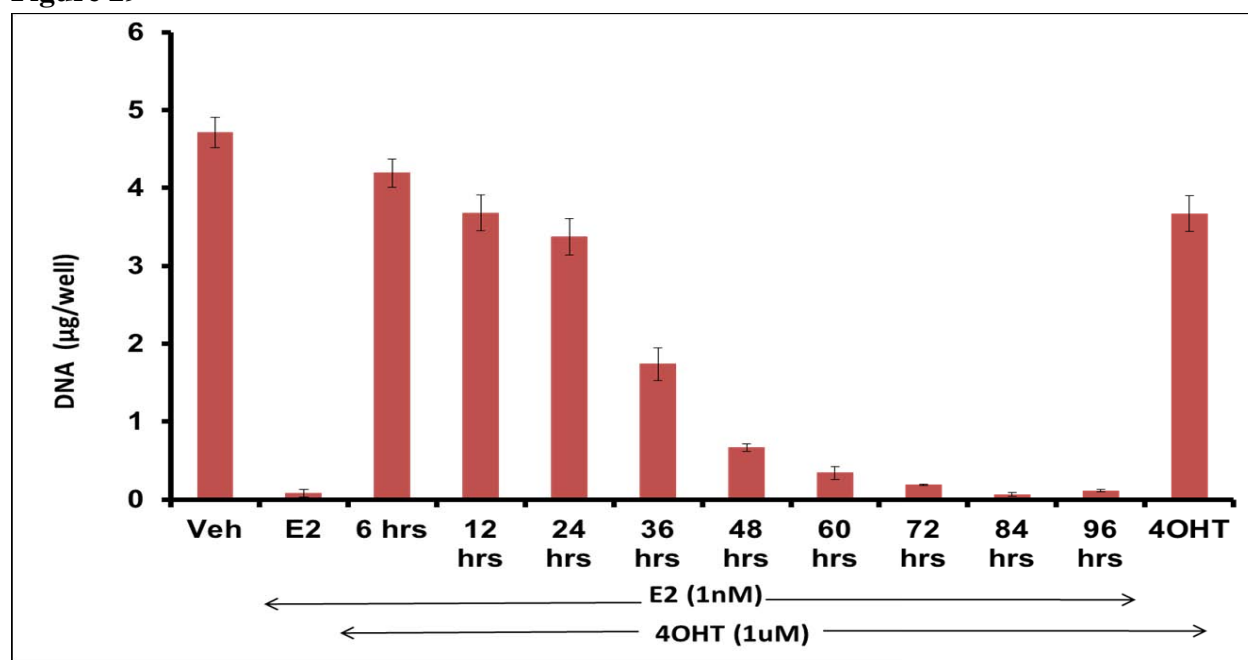
Figure 29

Figure 29. 4OHT fails to block E₂ induced apoptosis after 24hrs. 15000 MCF5C cells were seeded in 24 well plates in triplicates. Cells were treated with E₂ (1nM) alone and 1uM 4OHT was used to block E₂ treated cells at 6hrs, 12hrs, 24hrs, 36hrs, 48hrs, 60hrs, 72hrs, 84hrs and 96hrs. The extent of apoptosis was determined by measuring the DNA content of the remaining cells in each well. Each data point shown is average of 3 replicate +/- SD. E₂ mediated apoptosis cannot be rescued after 24hrs by 4OHT.

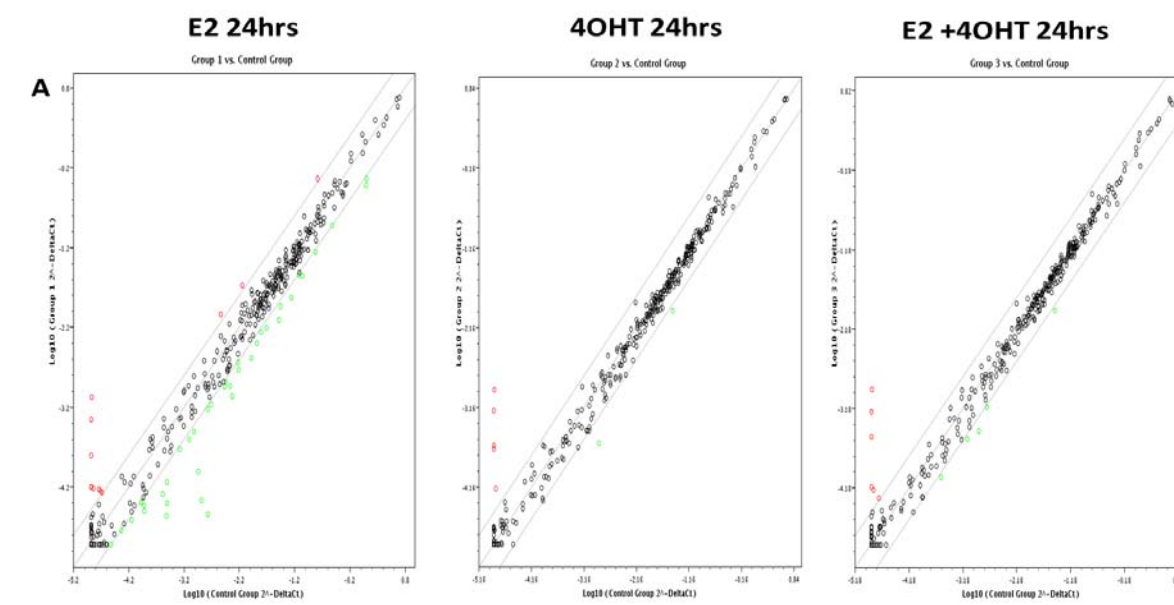
Differential gene expression of E₂ mediated apoptosis at the critical trigger point

To identify genes associated with E₂-induced apoptosis with a particular interest to the critical trigger time point, differential regulation of apoptotic gene expression in response to E₂ was interrogated in the MCF7:5C cells. Cells were treated with 1nM E₂ or without E₂ (control), 1uM 4OHT and E₂ in combination with 4OHT over a 48 hr time course consisting of 3 time points.

RNA was extracted and were quality controlled for expected induction of TFF1 (trefoil factor 1) mRNA expression (data not shown) in E₂-treated samples and for no induction in control-treated samples. Gene expression was measured using customized PCR arrays that include 384-well plate, for detection of apoptotic pathway focused genes as well as appropriate RNA quality controls. The PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray. Gene expression values were measured at 46 hr time point, as expected significant evidence of apoptotic gene induction is not apparent, rather proapoptotic genes appear to be differentially downregulated by E₂ (Fig. 30). However, PMAIP 1 (also known as NOXA) and Tumor necrosis factor super family members TNFRSF 8 and TNFSF 14 are upregulated. 4OHT, either in the presence or absence of E₂ is able to block E₂ mediated effects. Interestingly, at 36 hrs (Fig. 31), which represents the critical trigger point, E₂ induces proinflammatory genes such as CEBPB, endoplasmic reticulum stress (ERS) genes; DDIT3 which have previously been

reported in our global gene microarray study (9). BIM, DAPK1, NOXA and TP63, which all play a role in apoptosis, are upregulated. Expression of BIM correlates with the reported finding (8) of its protein expression in E₂ treated 5C cells and that SiRNA-mediated knock down of BIM prevented apoptosis. PMAIP 1 (also known as NOXA), a Bcl-2 homology (BH3) only family was expressed as early as at the 24hrs time point indicating that it may play a pivotal role in initiating the intrinsic pathway of apoptosis. Following 48 hrs of treatment (Fig. 32), the gene expression expands to involve p53 and death receptor genes FAS, TNFRSF21 and TNF and increased expression of the CEBPB family and the DAPK family. The identified apoptosis genes are listed in Figures 30C, 31C and 32C.

Figure 30



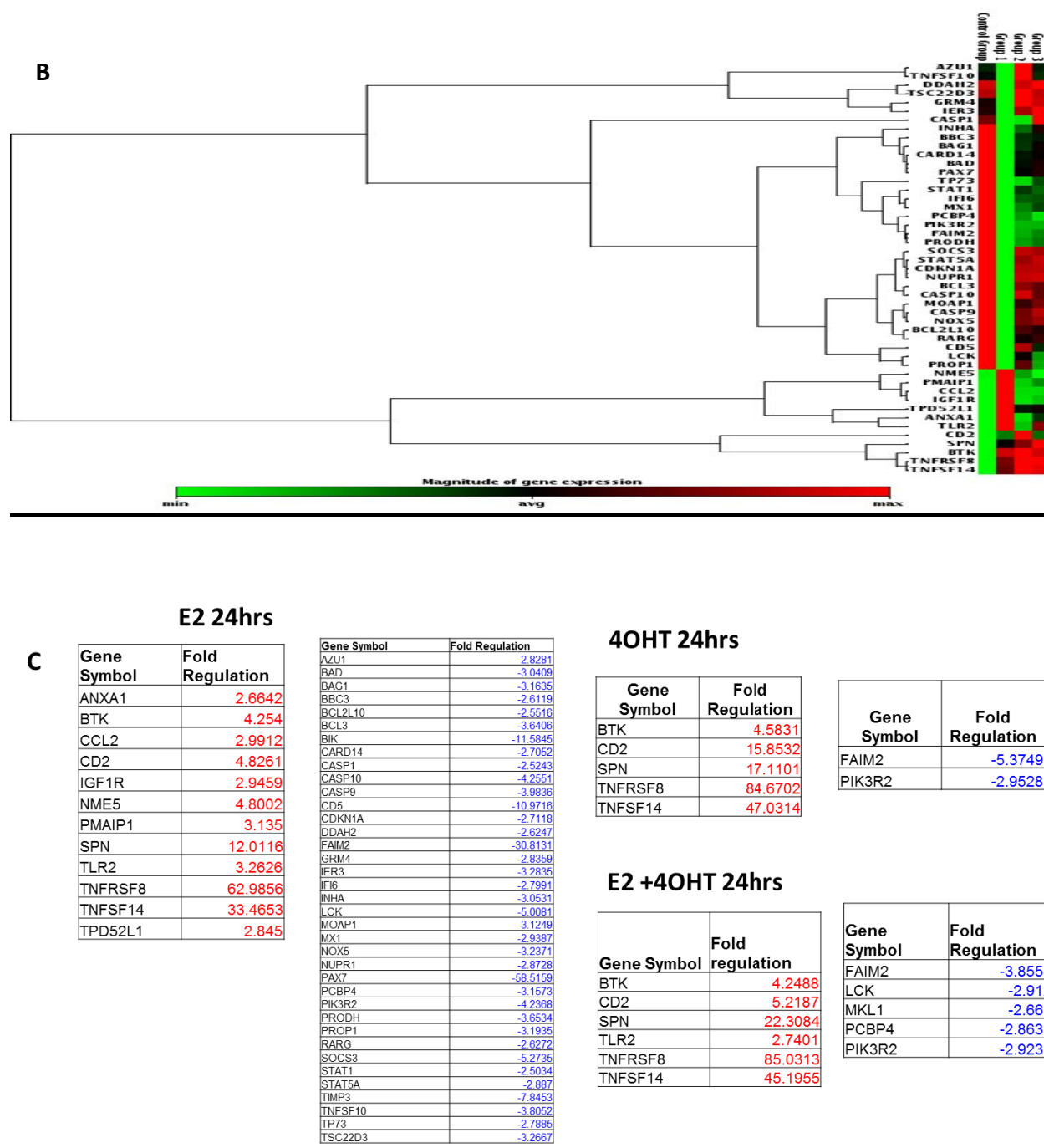
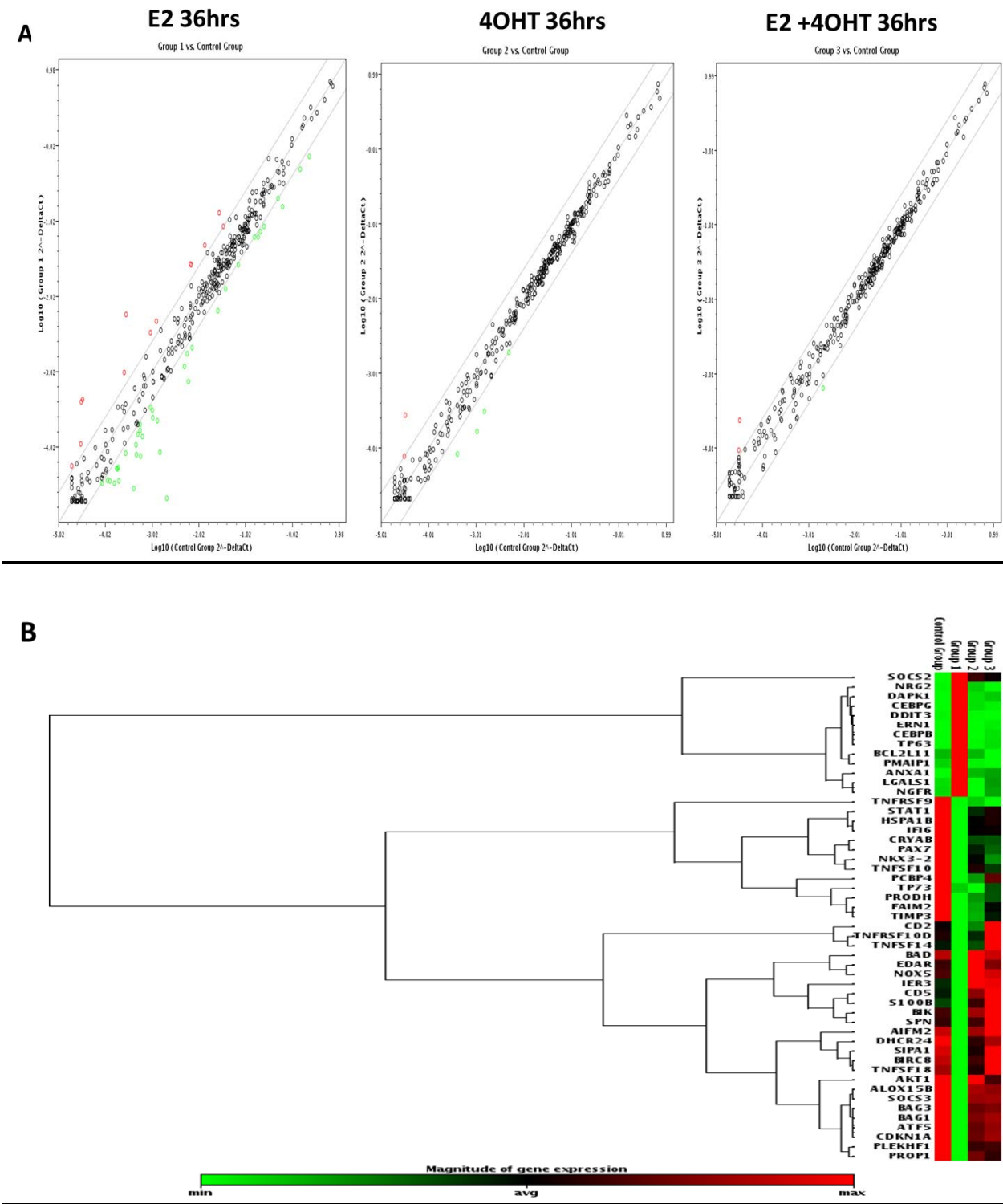


Figure 30. Determination of E₂ mediated apoptotic genes at its critical trigger point. MCF7:5C cells were seeded at 300,000 cells per well in 6 well plates, in estrogen-free media. Cells were parsed into groups of 3 replicates per treatment per time point, and then treated with either 0.1% ethanol (control), 1nM E₂, 1 uM 4OHT in the presence or absence of E₂, for 24, 36, and 48 hrs. Cells were harvested for RNA using TRIzol. Total RNA was isolated, quality controlled and reverse transcribed using a first strand synthesis kit. Samples were loaded onto customized PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analyzed in comparison to the controls at 24 hours (A, B, C). Genes upregulated are represented in pink and downregulated genes are represented in blue.

Figure 31



C E2 36hrs

Gene Symbol	Fold Regulation
ANXA1	13.087
BCL2L11	2.5359
CEBPB	3.8483
CEBPG	3.5422
DAPK1	2.9495
DDIT3	4.674
ERN1	3.6467
LGALS1	3.8284
NGFR	3.8614
NRG2	3.703
PMAIP1	4.0604
SOCS2	13.2718
TP63	20.7639

Gene Symbol	Fold Regulation
AIFM2	-3.2919
AKT1	-2.5341
ALOX15B	-3.3452
ATF5	-2.6333
BAD	-2.9942
BAG1	-4.0335
BAG3	-2.9004
NKX3-2	-3.3439
BIK	-4.4621
BIRC8	-4.7179
CD2	-4.2265
CD5	-3.5601
CDKN1A	-2.895
CRYAB	-5.5114
DHCR24	-3.0666
EDAR	-3.3839
FAIM2	-17.3873
HSPA1B	-4.0028
IER3	-3.0546
IFI6	-3.1681
NOX5	-3.4359
PAX7	-98.7112
PCBP4	-2.7309
PLEKHF1	-3.261
PRODH	-4.1825
PROP1	-3.5416
S100B	-3.2727
SIPA1	-5.8335
SOCS3	-8.0719
SPN	-2.7479
STAT1	-2.5343
TIMP3	-14.3925
TNFRSF10D	-3.0312
TNFRSF9	-2.526
TNFRSF10	-7.3991
TNFRSF14	-5.6928
TNFRSF18	-3.5206
TP73	-4.195

4OHT 36hrs

Gene Symbol	Fold Regulation
ANXA1	2.5671
SOCS2	8.5236

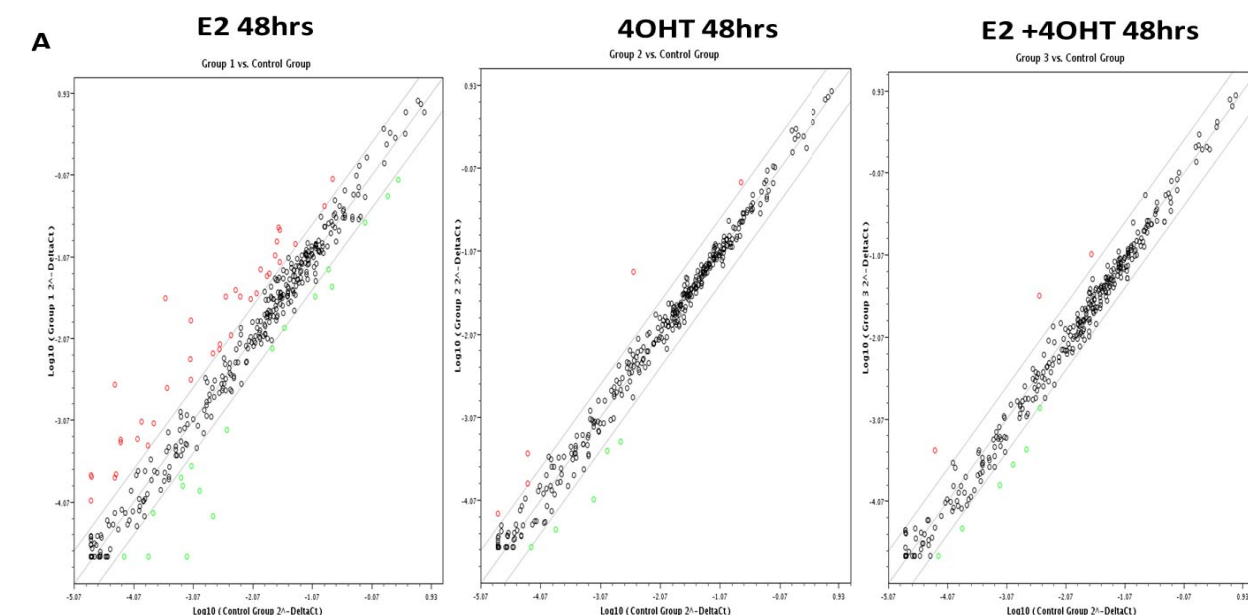
Gene Symbol	Fold Regulation
FAIM2	-4.7651
PRODH	-2.5397
TIMP3	-4.8255
TP73	-6.1608

E2 +4OHT 36hrs

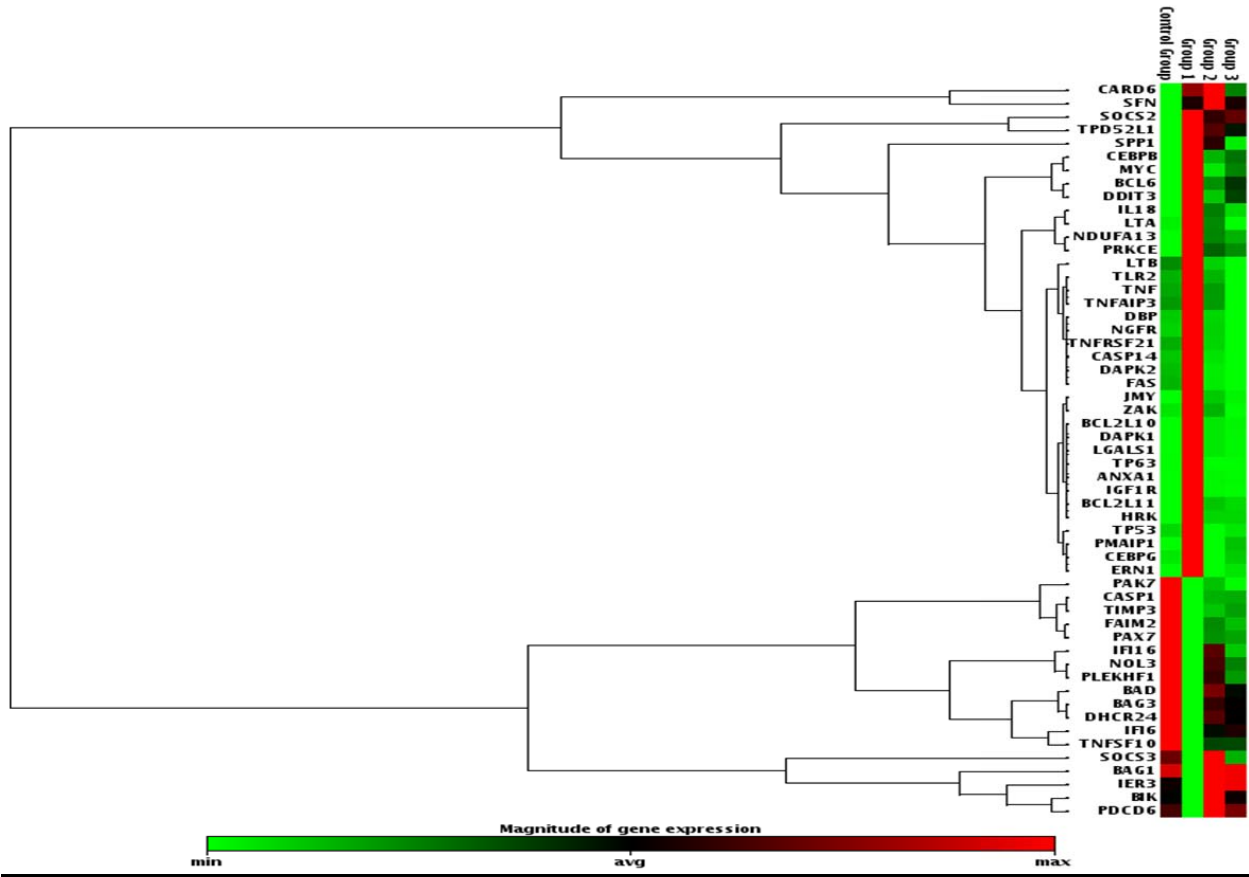
Gene Symbol	Fold Regulation
ANXA1	3.1103
SOCS2	7.4229

Gene Symbol	Fold Regulation
PAX7	-3.215

Figure 31. Determination of E₂ mediated apoptotic genes at its critical trigger point. MCF7:5C cells were seeded at 300,000 cells per well in 6 well plates, in estrogen-free media. Cells were parsed into groups of 3 replicates per treatment per time point, and then treated with either 0.1% ethanol (control), 1nM E₂, 1 uM 4OHT in the presence or absence of E₂, for 24, 36, and 48 hrs. Cells were harvested for RNA using TRIzol. Total RNA was isolated, quality controlled and reverse transcribed using a first strand synthesis kit. Samples were loaded onto customized PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analyzed in comparison to the controls at 36 hours (A, B, C). Genes upregulated are represented in pink and downregulated genes are represented in blue.

Figure 32

B



C	Gene Symbol	Fold Regulation
	ANXA1	57.3851
	BCL2L10	11.1112
	BCL2L11	6.1039
	BCL6	3.0659
	CARD6	5.1788
	CASP14	4.1381
	CEBPB	5.3823
	CEBPG	4.403
	DAPK1	10.1625
	DAPK2	3.3865
	DBP	3.2951
	DDIT3	8.5521
	ERN1	6.268
	FAS	3.1267
	HRK	3.3396
	IGF1R	5.1589
	IL18	2.7084
	JMY	7.5993
	LGALS1	18.2555
	LTA	8.9147
	LTB	2.8441
	MYC	2.8013
	NDUFA13	7.2126
	NGFR	6.8376
	PMAIP1	7.6316
	PRKCE	3.0529
	SFN	9.2554
	SOCS2	9.5122
	SPP1	5.5017
	TLR2	4.2949
	TNF	4.228
	TNFAIP3	3.0417
	TNFRSF21	2.5535
	TP53	2.612
	TP63	90.3002
	TPD52L1	4.1153
	ZAK	3.3906

BAD	-2.5023
BAG1	-2.7721
BAG3	-2.6497
BIK	-4.2268
CASP1	-8.1698
DHCR24	-4.8597
FAIM2	-9.287
IER3	-3.2977
IFI16	-2.8643
IFI6	-3.108
NOL3	-3.4184
PAK7	-3.222
PAX7	-32.2886
PDCD6	-2.8903
PLEKHF1	-3.4435
SOCS3	-4.8034
TIMP3	-35.754
TNFSF10	-3.1145

4OHT 48hrs

Gene Symbol	Fold Regulation
LTA	2.6067
SFN	15.8793
SOCS2	6.057
SPP1	3.6436
TPD52L1	3.0662

E2 +4OHT 48hrs

Gene Symbol	Fold Regulation
CASP1	-3.956
FAIM2	-3.2491
PAK7	-2.5648
PAX7	-4.2314
TIMP3	-7.6083

Gene Symbol	Fold Regulation
DDIT3	3.8055
SFN	9.1735
SOCS2	6.901

Gene Symbol	Fold Regulation
CASP1	-3.8153
FAIM2	-4.6224
PAK7	-3.2137
PAX7	-4.9887
SOCS3	-2.682
TIMP3	-4.8827

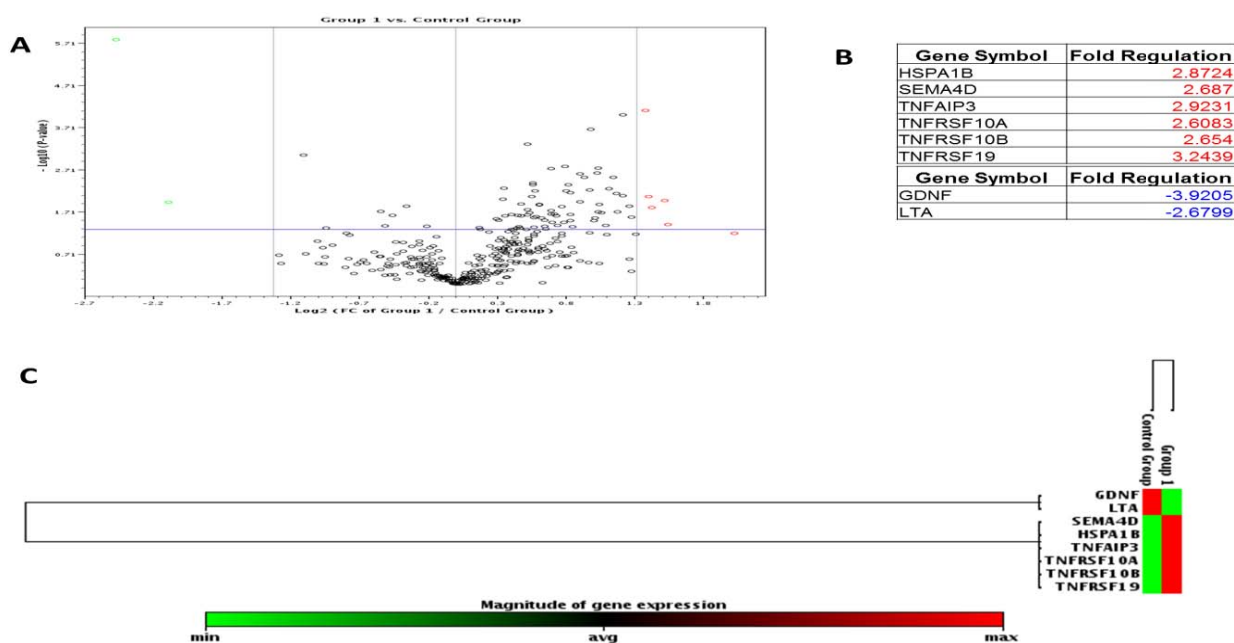
Figure 32. Determination of E₂ mediated apoptotic genes at its critical trigger point. MCF7:5C cells were seeded at 300,000 cells per well in 6 well plates, in estrogen-free media. Cells were parsed into groups of 3 replicates per treatment per time point, and then treated with either 0.1% ethanol (control), 1nM E₂, 1 uM 4OHT in the presence or absence of E₂, for 24, 36, and 48 hrs. Cells were harvested for RNA using TRIzol. Total RNA was isolated, quality controlled and reverse transcribed using a first strand synthesis kit. Samples were loaded onto customized PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analyzed in comparison to the controls at 48 hours (A, B, C). Genes upregulated are represented in pink and downregulated genes are represented in blue.

Paclitaxel induces apoptosis in MCF7:5C cells through a death receptor mediated pathway.

Because paclitaxel induces apoptosis rapidly in MCF7:5C cells, we further investigated expressed genes induced by paclitaxel that may elucidate a differential course of action. MCF7:5C cells were treated with 1uM paclitaxel at indicated time points and samples were quality controlled for gene expression using PCR array. In comparison to E₂, paclitaxel selectively activated the tumor necrosis factor (TNF) superfamily which represents a multifunctional proinflammatory cytokines involved in the regulation of a number of processes including apoptosis. Twelve hours (Fig. 33A, 33B and 33C) treatment induced TNFRSF10A tumor necrosis factor receptor superfamily, member 10a and TNFRSF10B which are known to be activated by the ligand tumor necrosis factor-related apoptosis inducing ligand

(TNFSF10/TRAIL), and causes death through the extramitochondrial pathway. Similarly an additionally expressed gene TNFRSF19 (tumor necrosis factor receptor superfamily, member 19) induces apoptosis in a caspase-independent manner. Furthermore death receptor genes FAS, TNF and other TNF super family genes; LTA, LTB are activated at 24 hrs treatment with paclitaxel (Fig. 33D, 33E and 33F) which further induces CDKN1A cyclin-dependent kinase inhibitor 1A (p21, Cip1) that is known to inhibit the activity of cyclin-CDK2 or -CDK4 complexes at G1 phase. DAPK1, NOXA, TNF and FAS are the genes induced by both E₂ and paclitaxel. DAPK1 activates gamma-interferon induced programmed cell death and its role in paclitaxel induced apoptosis yet to be determined.

Figure 33



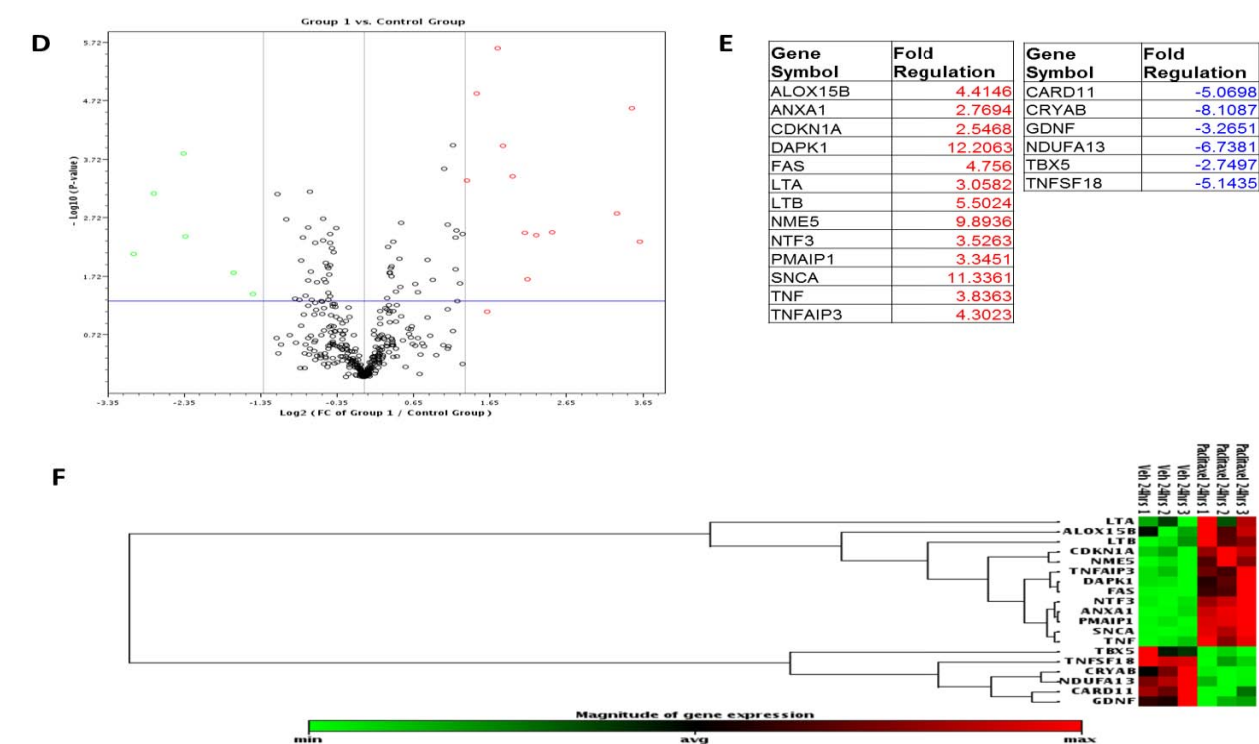


Figure 33. Determination of apoptotic genes induced by a cytotoxic chemotherapy in MCF7:5C cells. MCF7:5C cells were seeded at 300 000 cells per well in 6 well plates, in estrogen-free media. Cells were parsed into groups of 3 replicates per treatment per time point, and then treated with either 0.1% ethanol (control), or 1uM paclitaxel for 12hrs, and 48 hrs. Cells were harvested for RNA using TRIzol. Total RNA was isolated, quality controlled and reverse transcribed using a first strand synthesis kit. Samples were loaded onto customized PCR array plates with primers for indicated apoptotic genes. Gene expression values were obtained and analyzed in comparison to the controls at 12 hours (Fig. 33A, 33B and 33C) and 24 hours (Fig. 33D, 33E and 33F). Gene upregulated are represented in pink and downregulated genes are represented in blue.

Conclusion:

E₂-induced apoptosis occurs as a delayed event in MCF7:5C cells in contrast to the generally accepted norm. Paclitaxel, a cytotoxic chemotherapy, rapidly induces apoptosis in the same cell line by 24 hrs, while E₂ begins this process after 72 hrs using a cell proliferation assay. E₂ induces ERS and inflammatory stress genes as well as apoptotic genes that induce both the intrinsic and extrinsic apoptosis pathway. Given the above results, it is proposed that the delayed mechanism of apoptosis induced by E₂ involves an initial induction of both endoplasmic reticulum stress and proinflammatory stress with early involvement of NOXA and subsequent activation of mitochondrial mediated apoptotic genes that later expands to involve other apoptotic genes including the death receptor gene family. Therefore E₂-induced apoptosis involves a number of multifactorial events that may explain the delayed apoptosis that is observed in the MCF7:5C cells. On the other hand, paclitaxel selectively induces the TRAIL/TNFRSF10A/B pathway initially which expand to involve more death receptors with inhibition of the cell cycle at G1 checkpoint by p21. In addition, NOXA and DAPK1 also

kpf wegf "d{ "G₄ctg"gzr tguugf "cv'46"j tu0Vj g"ko r rdecvgf "tqng"qh'y gug"vy q"i gpgu"ku"pqv"qdxkqwn{ " cr r ctgpv"vq"y j g"tcr kf "cpf "f gnc{ gf "tgcvo gpv'y kj "r cerkcz gn'cpf "G₄"t gur gevkg n{ 0'
"
"

VCUM'5<'*I Wf lqi gn'cpf "Y gmmgkp+"/"Vq"f gekr j gt "egmwrt "uki pcrkpi "r cvj y c{ u'wulpi " r tqvgqo leu'cpf 'vq'b guj 'rtqvgqo leu'cpf 'b TPC'čpcrf uk0'

Kpvt qf wevkqp<'

Htqo "y j g"rtgxkqwn{ "r wdrkuj gf "gzr gtlo gpvu"qp"y j g"ej ctcevgtk vvkp"qh""y j g"OEH9<7E"egm' ewmwrtg""kp"xktq"y g"kf gpvkhgf "GT"cpf "CŒ3"kpvtcevki "r tqvgkpu"wpks wgn{ "eqpvtqmgf "kp"y j gk" kpvtcevkvpu"d{ "f hgtgpvkn'r cvgtpu"qh""r quv'vcpurcvkpcn'o qf hkecvkpu"*RVO u+"y j cv'kp"wt p"ecp" cevxcvgf "hgtgpv"uki pcrkpi "r cvj y c{ u'f qy pvtgco "qh'dqvj "qh'y go 0'

P gzv'y g"cko gf "vq"kf gpvkh{ "uki pcrkpi "gxgpv"y j cv'eqpvtqn'gustqi gp/kpf wegf "cr qr vquku"kp" xkxq"wo qt"o qf gn'cpf "eqo r ctg"y j ku'tguwn'y kj "qwt"rtgxkqwn{ "tgr qtvgf "cpf "r wdrkuj gf "tguwnu" htqo "kp"xktq"cpn{uku"y j cv'y gtg"r gthqto gf "kp"egm'ewmwrtg"*62-0'C"o qf gn'hqt"y j ku'ctg"wo qt" zgpqi tchn"qh"OEH9<7E"egm."c"nqpi /vgto "gustqi gp"f gr tkxvkqp/tgukncpv"xctkcpv"rkpg"qh'dtgcuv' ecpegt"OEH9"egm'y j cv'wvf gti q"cr qr vquku"kp"t gur qpug"vq"G₄0"

Y qtmiCeeqo r rkuj gf<'

Zgpqi tchn' wo qtu" y gtg" r tgr ctgf" kp" rcdqtcvqt{ " qh" Ft0' Iqtf cp0' Cyj {o k" pwf g" qxctkgevqo k gf "hgo crg"o keg"qh'6/7"y ggmu"qr"y gtg"r wtej cugf "htqo "J ctrcp"Ur tci wg"Fcy r g{ " *Hgf gtekem" O F +0' Hxg" O keg"y gtg" kplgev f "y kj "cdqw"7"o knkqp" OEH9<7E"egm"r gt" ukv." dkrvgtcm{ "kpq"y j g"o co o ct{ "hcv'r cf u'kp" c'3<3"o kzwtg"qh'i tqy y "hcevq'tgf wegf ."r j gpqn'tgf /"htgg" O cvki gn*"DF "Dkuekgpegu."Dgf hqtf ."O C+0Vj g"wo qt"etquu"ugevkpcn'ctgc"y cu'tgeqtf gf "y ggmu{ " wulpi "y j g"htqo wr<"}ngpi yj "eo +z"y kf yj "eo +z" 16_0'Ugxgp"y ggmu'chgt"klgevki "egm"kpq"y j g" o co o ct{ "hcv'r cf ."wo qt/dgctkpi "o keg'y gtg'tgcvgf "y kj "gkj gt'xgj keng"*322"wn'r gcpw"qkn"qt"402" o letqi tco u'qh'39/dgvc"gutcf kn'kp"322"wn'qh'r gcpw"qkn'd{ "kp'tcr gkqpgcn'kplgevkv0Vj g"gutqi gp" vgcvgf "wo qtu'y gtg"j ctxgugf "chgt"4"j qwtu"cpf "8"j qwtu"qh'tgcvo gpv0Vj g"wo qtu'y gtg'erncpgf " qh'cp{ "hcv'cpf "cf lqkpkpi "kuuwg."ur rk'kpq"vy q"r kgegu"cpf "upcr "htq| gp"kp"rks wkf "pktqi gp"cpf "ngr v" cv'/: 2 E"wpvkn'htv j gt "wug0""Qpg"j crh'y cu"wugf "hqt"r tgr ctcevkv"qh'htq| gp"ugevkpu"cpf "J (G" ucklpki 0Vj g'tguv'y cu"wugf "hqt"r tqvgkpu"cpf "TP C"gzvcevkv0"

Vj g"r ctchhkp"etquu"ugevkqp"qh"wo qt"uco r ngu"y gtg"uckpgf "y kj "cp"J (G"cpf "uj qy gf " egmwrt"j qo qi gpvkv{ "cpf "wo qt"eqpvgp"y kj "o qf gtcvg"eqppgevkg" kuuwg"eqpvgp" cpf "rkvwg" pgetquku"*fcv"pqv'uj qy p+0'

Vj g"r cvgt p"qh"G₄f gr gpf gpv'tcpuetkr vkp"cevxcvkqp"kp"OEH9<7E"wo qt"zgpqi tchn'y cu' cuuguugf "d{ "s TV/RET"cpf "eqo r ctg"y j kj "c"tgegpv"kp"xktq"uwf { "*, +0'" "Vqcn"TP C"htqo "y j g" wo qtu'y cu'kuqrcvgf "wulpi "VTKqn'tgci gpv"*Kpxktqi gp."Ectndcf ."EC."WUC+"cpf "cp"TP gcu{ "nk' "S kci gp"Kpe0"cu"r gt"y j g"o cpw'cewtgr'u"kpvt wevkpu0' Qpg""wi "qh'y j g"vqcn"TP C"y cu'tgxgtug" vcpuetkdgf "kp" c"vqcn'xqnw g"qh'42"wn" wulpi "c"j ki j /ecr cekv{ "eF P C"tgxgtug"vcpuetkr vkp"nk' "Crr rkgf " Dkqu{ugv u." Hqugt" Ek{." EC." WUC+ "cu" r gt" o cpw'cewtgr'u" kpvt wevkpu" cpf " uwdugs wgpvn{ "f knwgf "vq"722"wn'y kj "uvgtkrg"y cvgt0Vj g'tgcn'vko g"RET"y cu'r gthqto gf "kp" c"42"wn"

tgcevkqp"y j lej "lpenwf gf "3zU DT"i tggp"RET"o cuxgt"o lz"*Crr rkgf "Dkqu{ ugo u:"347"pO "gcej "qh" hqty ctf "cpf "tgxgtug"r tko gtu"cpf "7"vN"qh"l kwwgf "eF P C" wulpi "cp"CDKRtkuo "9; 22"J V"Ugs wgepg" F ggevkqp"U{ ugo "*"Crr rkgf "Dkqu{ ugo u+"hqt"62"e{ ergu"; 7^qE "hqt"37"u."82^qE "hqt"3"o kp+"hqmjy kpi " cp"lplkcn"32"o kp"lpewdcvkp"cv"; 7^qE0Vj g"lqrf "ej cpi g"lp"gzr tguukqp"qh"tcpuetr u'y cu"ecrewwvgf " wulpi "y g"gf gnc/f gnc"Ev'o gyj qf."y kj "y g"tkdquqo cnr tqvklp"58D6"o T P C"cu"y g"lpvgtpcn'eqptqrf" Vj g'r tko gt "ugs wgepgu"wgf "ctg"cu"lqmjy u<"

58D6<Hy f '7'GVI VVEI CECCVI I ECI EAT3'="

58D6<Tgv 5'GCECEEEVEECI I CCI EI C3'="

E/O[E<Hwd5'I EECEI VEEVEACACATCAG3'="

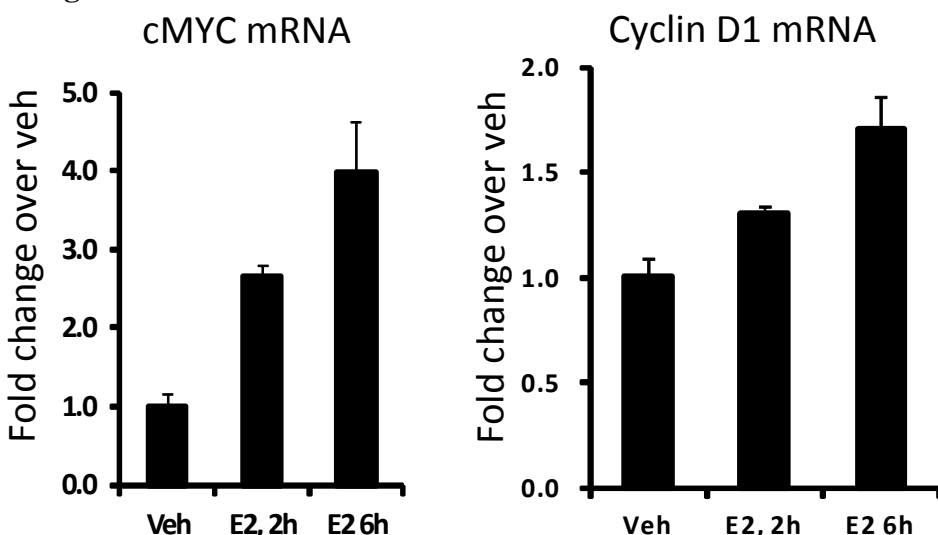
E/O[E<Tgv 5'TEVI I ECI ECI I CVCI VEETT3'="

E{enp"F3<Hwd 5'VCVI EI EVI EVCEEI VVI C3'="

E{enp"F3<Tgv 5'CCCCVCI ECI ECCCECCVI VI CCC5'."

Vj g"tcpuetr vkp"cevkkl"qh"y tgg"y gni"mpqy p"gutqi gp"tgi wcvgf"i gpgu."pco gn{ "eO[E." E{enp"F3"cpf "RU4"*VHB+y gt g"lpf wegf 0Vj g'o quv'r tqo kpgpv'ghge'v'qh"gutqi gp"y cu"qdugtxgf "lp" eO[E"tgi wcvkp."lqmjy gf "d{ "e{enp"F3"cpf "RU4"*Hi 0'56=f cvc"uj qy p"lqt"eO[E"cpf "E{enp" F3+0Qxgtcm"y gug"fcv"ctg"lp"ceeqtf"y kj "y g"lp"xkt q"tgr qt v'cpf "f qgu'r tqxkf g"gxkf gpeg"qh"39 " gutcf kqf'o gf kcvgf "tcpuetr vkpcn'cevkcvkp"lp"y g"zgpqi tch'wo qtu"chgt"uj qt v'vgo "4"j tu"cpf "8" j tu"qh"vgtqkf "tgcvo gp0" "

Hi wt g'56"



Hi wt g'560Tgi wcvkp"qh"gutqi gp"t gur qpukg'i gpgu'lp'wo qt "zgpqi tchu"O EHD<7E+d{ "G4"chgt "4"j tu'cpf "8" j tu'xgtuwu'xgj keng0F cvc"r tguipygf "lp"lqrf u"qh"lpf wevkqp"qh"tcpuetr vkp"qh'o T P C"lqt"eO[E"cpf "E{enp"F3"i gpg" qxgt"xgj keng'lgxgr0""

Cpcn{uku'qhiG4/lpf wegf 'r j qur j qv{ tqupg'r tqvklp'eqo r ngzgu'lp'O EHD<7E'wo qt 'zgpqi tchu0"

Vj g"tguv'qh'wo qt "zgpqi tchu'y gt g"n{ ugf "lp"PR/62"n{uku'dwhgt"*72"o O "Vtku."r J ": Ø."372" o O "P cEn"62"o O "d/i n{egtqr j qur j cvg."2047" "uqf kwo "f gqz {ej qrcvg."3" "PR/62."72"o O "uqf kwo " hmqtkf g."42"o O "uqf kwo "r {tqr j qur j cvg."3"o O "GI VC."3"o O "uqf kwo "qt vj qxcpcf cvg."cpf "

32wnlo n'r j qur j cvg"kpj kdkqt"eqemckn'3."*Uki o c+"wulpi "cdtcukxg"I tggp"Dgcf u"lp"O ci P c"N{ugt" j qo qi gpk gt"ceeqtf lpi "v{y g'o cpwlcwgt's prqvceqf0"

Y g"r gthqto gf "r j qur j qv{tqulpg"ur gekhe"ko o wqpr tgekr kcvkp"qh"n{ucvgu"htqo "wvpgcvgf" cpf "G4"tgcvgf "4j "cpf "8j "+"wo qt"zgpqi tchv"lqmny gf "d{"tguqmkpi "qp"r tgekr kcvgf "r tqvgkp"qp" UF U/RCI G"Hi 057+0Vj g"n{ucvgu'y gtg"r tgergtgf "y kj "52w/qh'Rtqvkgp"C"ci ctqug"dgc f u"htq"3j ." 6E"qp"c"tqemkpi "r rchqto "cpf "y g"tgs vktgf "co qwpv"qh'r tqvgkp"*5o i +"y cu"eqo dlpgf "y kj "6I 32/ eqplwi cvgf "qt"eqttgur qpf lpi "cpvkdqf lgu"cu"f guetldgf "r tgxkqwn{ "62+0Wpeqplwi cvgf "cpvkdqf lgu" y gtg"r tgekr kcvgf "y kj "r tqvgkp"I "dgc f u"*Tqej g+0K o wpqeqo r rgzgu'y gtg"y cuj gf "7"vko gu'y kj " 207" " " P R/62 IRDU." dqlkrf " kp" NF U/uco r rg" dwhgt." cpf " tguqkxgf " qp" UF U/RCI G" *P wr ci g." kpxktqi gp+0"

Eqqo cuukg" Dnwg" uvcplpi " qh" UF U/RCI G" uj qy gf " utqpi guv" ceewo wrcvqp" qh" r j qur j qv{tqulpg"cuuqekcvgf "r tqvgkp"chgt"4j "Hi 057."ugeqpf "rcpg"kp"uvcplgf "i gn:"y j gtgcu"y g"8j " htcevqp"s wpej gf "vq"cm quv'c"s vkguegpv'uci g"*rkng"xgj keng/qpn{ +0Vj gtghgtg."hqt"htv j gt"uwf {"y g" ej qug"4j "zgpqi tchv'wo qt"htq"O U"pcn{ uku'0Cm'f khtg pvcn'cpf "gpj cpegf "dcpf u"qh'rcpg"y kj "4j " G4" r wmf qy p" y gtg"gzekugf "vqi gj gt"y kj " y g"eqttgur qpf lpi " tgi kqp" qh" y g"cf lcegpv' rcpg" qh" xgj keng/vtgcvgf "wo qtu"*xgj keng"qpn{ +0Nqecvqp"qh"gzekugf "dcpf u"vqi gj gt"y kj "eqttgur qpf lpi " tgi kqp"kp"c"i gn'qh'y g"xgj keng/qpn{ "r wmf qy p"ctg"f gr kvgf "qp"Hi 0570"

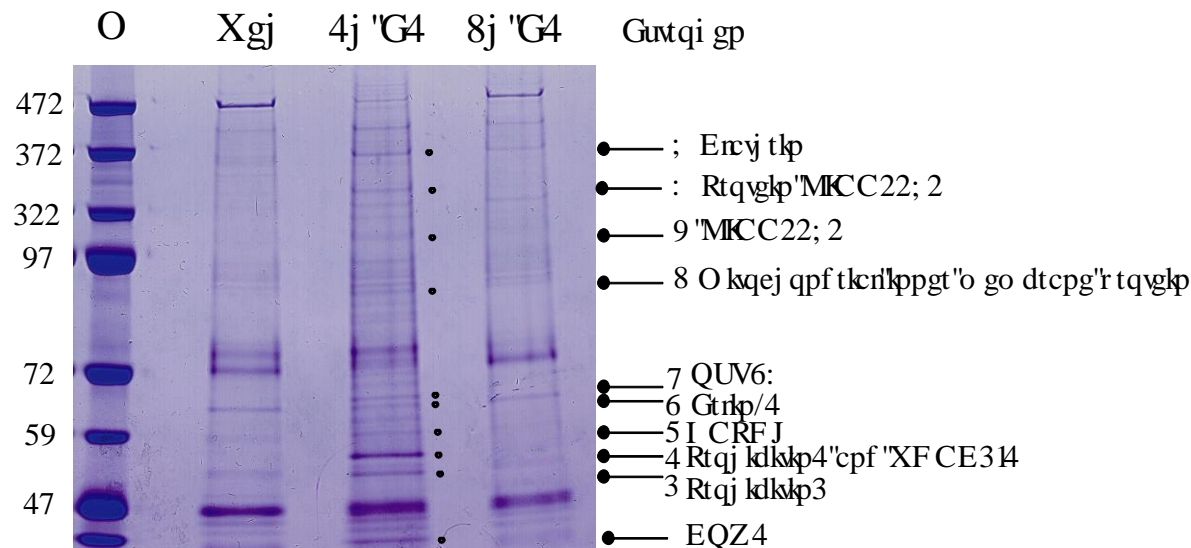
Vj g"eqttgur qpf lpi "dcpf u"y gtg"ew"qww"e{uvgkpgu"y gtg"tgf wegf "y kj "F VV."lqmny gf "d{" cm{rcvqp"y kj "kqf qcegwo kf g0'kp/i gn'f ki gukqp"y cu"r gthqto gf "qxgtplj v'd{"v{r ulp"gp{ {o gu0' Rgr vlf g'o kzwgu'y gtg"htcevqpvcvgf "wulpi "c'tgxgtugf "r j cug"eqnwo p"DGI 'E3: "eqnwo p"*30'Üo ."97" Üo "z"372"o o ."Y cvgtu+"qp"pcpqWRNE"Ces vkw{"*Y cvgtu+"y kj "dwhgt"C"*4" "cegvpktkrg."208' " hqto le"cekf "+"cpf "dwhgt"D"; : ' "cegvpktkrg."208' "hqto le""cekf +0Vtkr vkr"r gr vlf gu'y gtg"gnwgf " qxgt"52"o kp'rkpgct"i tcf kgpv'qh'2/82' "qh'y g'uqkxgpv'D'y kj "522'pnlo kp'hty 'tcvg0Vj g'pcpqWRNE" kputwo gpv'y cu"eqwr nrf "vq"S UVCT"Grkxg"S Vql"*wcf twr qrg hko g/qh/hki j v"htq"tgi wrct"NE/GUK O U" cpf " kphqto cvkqp" F gr gpf gpv' Ces wukukqp" *FC+" qt" vq" 6222S VTCR" j {dtkf " vkr rg" s wcf twr qrg lrpget" kqp" vcr " o cuu" ur gestqo gvgtu" hqt" O TO /kpkkcvgf " f gvgevkqp" cpf " ugs wpekpi " *O FC U+0Dqy "kputwo gpv'y gtg"gs vkr r gf "y kj "P cpqUr tc{ "Kkqpquwteg"*Cr r rkf "Dkqu{uogo u." Hico kpi j co ."O C+0O UIO U'htci o gpv'kqp"fcv'y gtg"ugctej gf "ci clpuv'c"j wo cp"fcvdcug"wulpi "y g" O cueqv'cni qtkj o "O cvtkz"Uelgpegu."Nqpf qp."WM+"cpf "Rctci qp"cni qtkj o "kp"RtqvkgpRkqv"502" uqhy ctg"*CD"Uelgz+"y kj "o cuu"vqrgtcpeg"htq"r tgewtuq"lqpu"204'F c."htci o gpv'kqp"vqrgtcpeg"204" F c."qpqg"o kuu"ergxcig."ectdqz {o gj {rcvqp"cu"hzgf "o qf khtcevqp" hqt" e{uvgkpg."cpf "ugxgtcn' xctkdrng" o qf khtcevqp" uwej " cu" qzlf cvkqp" hqt" o gj kqplpg" cpf " r j qur j qt {rcvqp" hqt" ugtkpg." y j tgqplpg"cpf "v{tqulpg0"

Cm'r tqvgkp"y kj "j ki j guv'eqxgtci g"cpf "ueqtg"cuuki pgf "d{"RtqvkgpRkqv"fcvdcug"ugctej " uqhy ctg"cpf "cv'rgcu"6'f gvgevgf "r gr vlf gu'y gtg"i tqwr gf "kp"c"vdrng"d{ "y g'r qukkqp"qh'dcpf u"y j gtg" y j g{ "y gtg"f gvgevgf 0Rtqvkgp"htqo "cf lcegpv'dcpf u"qh'wpuko wrcvgf "wo qtu"*xgj keng"vgtcvgf "+"y gtg" uwdtcevgf "htqo "c"i tqwr "qh'G4/kpf wvdrng"r tqvgkp"0F cvc"ctg"r tgugpvgf "kp"c"vdrng"vqi gj gt"y kj "c" r tqdcdkx{ "ueqtg."y j ksj "y cu"cuuki pgf "d{" RtqvkgpRkqv"vqcn'eqxgtci g" qh" r tqvgkp"y kj " y j g" pwo dgtu"qh" f gvgevgf "r gr vlf gu."ceeguukqp"pwo dgt"qh"eqttgur qpf lpi " r tqvgkp" cpf " r qukkqp" qh" eqttgur qpf lpi "dcpf u"y j gtg"r tqvgkp"y gtg" f gvgevgf 0' "kp"cf f kkkqp."r qukkqp"qh"gzekugf "dcpf u"

qvi gvj gt'y kj "o cvej kpi "tgi kqpu"kp"vj g"i gn'qh"vj g"xgj kerg/qpn"r wnf qy p"ctg"f gr kevqf "qp"vj g"i gn' ko ci g"Hi wtg'57-0'

"

Hi wtg'57"



"

Hi wtg' 570' NE/O U' cpcnf uku' qh' cpwkrj qur j qv tqukpg' *cpwkr [+ ' rwnqf "f qy p" r tqvqkpu' ltqo " G4/vt gcvqf " OEH9-7E'wo qt 'zgpqi tchud' Tgr tguqpcvkg'ucvqpf "i gnu'ht'r j qur j qv tqukpg'r tqvqo g'cpcnf uku'Rtqvqk'gzvcew' ltqo "G4-qt"xgj kerg"tgcvgf "OEH9-7E'wo qtu'y gtg'ko o wqqr tgekr kevqf "y kj "6I 32'cpwkr ["eqplwi cvqf "cpvldqf {0Cm' f lhtgtpvkn'cpf "gpj cpegf "dcpf u'y gtg"gzekugf "ltqo "i gnu'qvi gvj gt'y kj "vj g"eqttgur qpf kpi "dcpf "kp"vj g"xgj kerg/qpn" r wnf qy p"cpf "cpcnf | gf "d { "NE/O WOF qo kpcpv'r tqvqkpu"kp" gcej "dcpf "qh"vj g"rki cpf / "uko wcvqf "rcpg"qh"vj g"i gn'ctg" kpf kevqf 0Rtqvqkpu"kp"vj g"eqttgur qpf kpi "dcpf "ltqo "vj g"xgj kerg/qpn" rcpg"y gtg"uudvcevgf 0Vj g"hwri'ku'qh'r tqvqkpu"ku' uj qy p"kp"Vcdrg'50'

"

Qxgtcm'y g"lf gpvklgf "68'r tqvqkpu"*Vcdrg'5-0'Co qpi "vj go "ctg'37"o kqej qpf tkcn'r tqvqkpu' Qpg" i tqwr " qh" vj gug" o kqej qpf tkcn' r tqvqkpu." vj g" xqnci g/f gr gpf gpv' cplqk/ugrgevkg" ej cppgr' r tqvqkpu"*XF CEu+."CFRICVR"vcpunqecugu"*CP Vu+."cpf "W/v{r g"o kqej qpf tkcn'etgcvqkpg"nkpcug" *wO vEM+."hqtu u" c" Rgto gcdkrk{ " Vtcpuklqp" Rqtg" *RVR+ "eqo r rgz" y j lej " tgi wcvgu"CFRICVR" gzej cpi g"dgvy ggp"vj g"o kqej qpf tkcn'o cvtkz"cpf "vj g"e { vqr nuwo " *ugg"ectvqpp"kp"Hi 0'58-0'Vj g" hwpvqpcn'ucwuu"qh'RVR"r n { u"cp"ko r qvcpv'tqrg"kp"vj g"lpkcvqk"qh'cr qr vuku"cpf "eqpvtnu"vj g" r gto gcdkrk{ "qh"vj g"kppgt"o kqej qpf tkcn'o cvtkz0'Qvj gt"r tqvqkpu."hwpf "kp"vj ku"gzr gtko gpv."rkng" o kqej qpf tkcn'r tqj kdkpu"qt"i n'egtrf gj {f g/5/rj qur j cvg'f gj {f tqi gpcug"*I CRF J +."kpvgtcev'y kj " c" f lhtgtpv'eqo r qpgpv'qh'RVR"eqo r rgz " *Vcdrg'5-0"

"

"

"

"

"

"

"

Vcdhg'5"

Ueqtg"	' " Eqx"	Ceequukp'%"	P co g"	Rgr wf gu"	Dcpf "
5804"	: 50 "	ur -R57454-RJ DaJ WO CP "	Rt qj kllslp"	52"	3"
420; "	6208"	ur -R27363-CF V4aJ WO CP "	CFRICVR'tcpunecug'4'*CPV4+'	33"	3"
3: 0; "	5: 08"	ur -R34458-CF V5aJ WO CP "	CFRICVR'tcpunecug'5'*CPV5+'	32"	3"
320 9"	4: 04"	ur -S 22547-0 RERaJ WO CP "	Rj qur j cvg'ectt lgt 'rt qvqlp.'b lskqj qpf tlcrl'	9"	3"
32048"	4808"	ur -S 24; 9: -0 4QO aJ WO CP "	O lskqj qpf tlcrl4/qzqi nwet cvglb crvg" ectt lgt 'rt qvqlp"	7"	3"
: 0 7"	5305"	ur -S ; R2N2-XCRCaJ WO CP "	Xgukeng/cuuqekcvgf "o go dtcpg'rt qvqlp/ cuuqekcvgf 'rt qvqlp"C"	7"	3"
: 084"	550 "	ur -S 36387-0 NGEaJ WO CP "	O crgevlp"	6"	3"
9088"	440 "	ur -S ; [8E; -0 VEJ 4aJ WO CP "	O lskqj qpf tlcrl ectt lgt 'j qo qmji '4"	6"	3"
5602: "	: 305"	ur -R439; 8-XF CE3aJ WO CP "	Xqnci g/f gr gpf gpv'cpkqp/ugrgevlsg'ej cppgjl' r t qvqlp'3'*XF CE3+'	54"	4"
4; 076"	8; 04"	ur -S ; ; 845-RJ D4aJ WO CP "	Rt qj kllslp/4'*RJ D4+'	4: "	4"
44067"	8204"	ur -R67: : 2-XF CE4aJ WO CP "	Xqnci g/f gr gpf gpv'cpkqp/ugrgevlsg'ej cppgjl' r t qvqlp'4'*XF CE4+'	39"	4"
37093"	7404"	ur -S 37228-VVE57aJ WO CP "	Vgvtcvleqr gr wf g'tgr gcvt'rt qvqlp'57"	9"	4"
33096"	460 "	ur -S ; P Y U: -TOPF 3aJ WO CP "	Tgs vktgf 'hqt'o glqvlb'pwenct'f kxkukqp'rt qvqlp'3" j qo qmji "	8"	4"
3309"	5904"	ur -R85466-I DNRaJ WO CP "	I wcpkpg'pwenqwf g/dkpf lpi 'rt qvqlp'lw dwpk/ dgvcl/4/rkng'3"	8"	4"
32063"	4804"	ur -S ; R257-J CEF 5aJ WO CP "	5/j {ftqz{ce{nEqC'f gij {ftcvug"	7"	4"
32084"	580 "	ur -S ; 8CI 6-NTE7; aJ WO CP "	Ngwelpg/tlej 'tgr gcveqpcwklpi 'rt qvqlp'7; "	7"	4"
6"	4; 09"	ur -S ; [499-XF CE5aJ WO CP "	Xqnci g/f gr gpf gpv'cpkqp/ugrgevlsg'ej cppgjl' r t qvqlp'5'*XF CE5+'	6"	4"
4502; "	7; 09"	ur -R266284 5RaJ WO CP "	I n'egtcif gj {f g/5/rj qur j cvg" f gj {f t qj gpcug'*1 CRFJ +'	47"	5"
3908"	7804"	ur -S ; ; 845-RJ D4aJ WO CP "	Rt qj kllslp/4'*RJ D4+'	: "	5"
3408"	5306"	ur -R44848-TQC4aJ WO CP "	J gvgtqi gpgqwu'pwenct'tldqpwenqqr t qvqlpu" C4ID3"	8"	5"
33046"	4: 0 "	ur -R29; 32-J PTREaJ WO CP "	J gvgtqi gpgqwu'pwenct'tldqpwenqqr t qvqlpu" E3IE4"	8"	5"
38"	5308"	ur -Q; 6; 27-GTNP 4aJ WO CP "	Gtrlp/4'*GT'hr kf 'tchv'rt qvqlp+'	: "	6"
33029"	520 "	ur -Q; 822: -VQO 62aJ WO CP "	O lskqj qpf tlcrlilo r qt v'tgegr vqt 'lwdwpls'	9"	6"

			VQO 62'j qo qni "		
:	37Œ"	ur -R73; ; 3-TQC5aJ WO CP "	J gvtqi gpgqu'pwerct'ldqpwerqr tqvlp "C5"	7"	6"
48"	750"	ur -R5; 878-QUV6: aJ WO CP "	F qrlcj {nfrj qurj qqri quceej ctkf g/r tqvlp" i n{equ{ntcpuhgtcug'6: 'nFc"	43"	7"
3; 074"	630"	ur -S 34; 27-KH4aJ WO CP "	lpvgtngwlp'gpj cpegt/dlpf lpi 'hrevqt"4"	32"	7"
3206"	4904"	ur -R46974-VJ K&aJ WO CP "	Cegv nEqC'čegv nt cpuhgtcug." o lqej qpf tkci'	7"	7"
: 074"	520"	ur -S ; J EW7-RTGDaj WO CP "	Rtqrvekp'tgi wrvqt { "grgo gpv/dlpf lpi 'rtqvlp"	6"	7"
8"	4: 0 "	ur -R34754-METWaj WO CP "	Etgcvpq'hlpqg'Wv'rg'b lqej qpf tkci' *wO vEM#"	6"	7"
45087"	4: "	ur -S 38: ; 3-ŒO O VaJ WO CP "	O lqej qpf tkci'lpqgt'b go dtcpg'rtqvlp"	33"	8"
39089"	45"	ur -R249: 8-VHf3aJ WO CP "	Vtcpuhtlp'tgegr vqt'rtqvlp"3"	:	8"
3808"	4204"	ur -R38837-CV4C4aJ WO CP "	Ucteqr ncuo le lgpf qr ncuo le'tgkwnwo 'ecrekw " " CVRcug"4"	;	8"
38029"	380"	ur -R45468-UHRS aJ WO CP "	Ur nlepi 'hrevqt.'rtqrlpg/'cpf'i nwoo lpg/tlej "	:	8"
3708: "	4904"	ur -R2: 45: J U; 2DaJ WO CP "	J gcv'uj qenir tqvlp"J UR"; 2/dgc"	8"	8"
; 087"	; 0"	ur -R27245-CV3C3aJ WO CP "	Uqf kwo lr qvcukwo /vcpur qtlpi 'CVRcug" uudwplk/cnrj c/3"	6"	8"
9084"	: 04"	ur -S ; PT52-FFZ43aJ WO CP "	P wengqrct'TP C'j grlecug"4"	6"	8"
905"	:	ur -S 35946-QI Uaj WO CP "	Ocppqu{nqri quceej ctkf g'i nvequlfcug"	6"	8"
34056"	380"	ur -R29; 22-J U; 2CaJ WO CP "	J gcv'uj qenir tqvlp"J UR"; 2/cnrj c"	7"	8"
90: "	3708"	ur -Q36; : 5-CV4C3aJ WO CP "	Ucteqr ncuo le lgpf qr ncuo le'tgkwnwo 'ecrekw " " CVRcug"3"	8"	8"
73069"	6808"	ur -S : P 988-M22; 2aJ WO CP "	Wpej ctcevgtk gf'rtqvlp"MKCC22; 2"	4: "	9"
3: 0 "	430 "	ur -S 2443: QF Q3aJ WO CP "	4/qzqi nwctcvg'f gj {ftqi gpcug." o lqej qpf tkci'	;	9"
35028"	4504"	ur -S : P 988-M22; 2aJ WO CP "	Wpej ctcevgtk gf'rtqvlp"MKCC22; 2"	7"	:
34058"	3408"	ur -S ; [8M7-QCU5aJ WO CP "	4/7/qri qcf gp{rvg'u{py cug"5"	8"	:
3208"	90"	ur -S 2: 433-FJ Z; aJ WO CP "	CVR/fgr gpf gpv'TPC'j grlecug"C"	7"	:
8608: "	5: 0"	ur -S 22832-ENf 3aJ WO CP "	Encvj tlp'j gcx{'ej clp"3"	53"	;
36064"	520"	ur -R43: 32-RI U3aJ WO CP "	Dli n{ecp"	9"	;

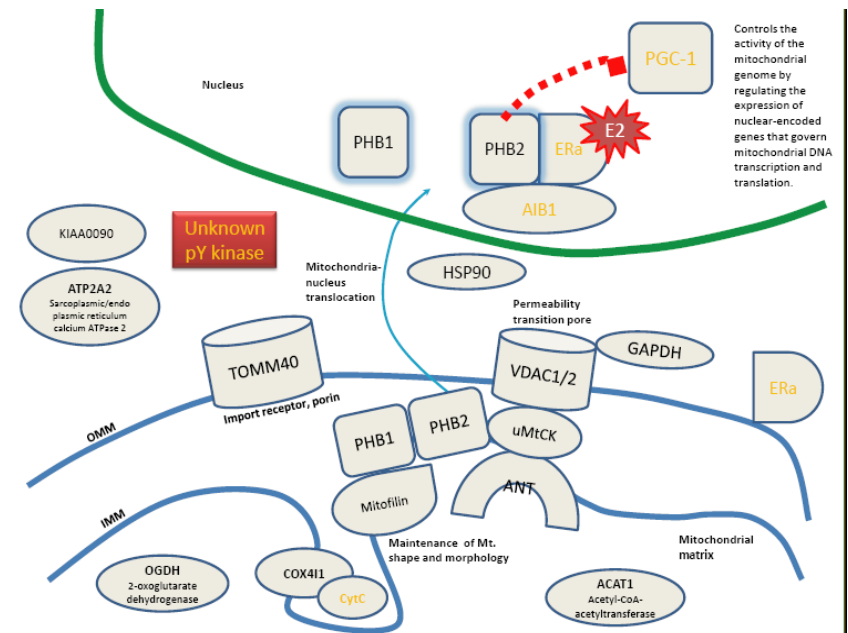
"

Vcdrg'50'G/lpf wkdrg'rj qurj qv' tqupg/cuqekvgf 'rtqvlp'u'x[/rtqvqgo g+'lp'O EHD-7E'wo qt'zgpqi tchul'
Rtqvlp'u"y gtg"r wngf" f qy p"cu"rj qurj qv' tqupg"cuqekvgf" eqo "n rnzgu"htqo "n{ucv'u"qh"vj g"zgpqi tch"wo qtu"qh"
gustqi gp'tgcvgf "o leg"*4j "chgt"gutqi gp'lp'lgv'kp+0Rtgekr kcv'gf 'rtqvlp'u"y gtg'tguqrgf "qp"UF U/RCI G'*Hli 057+cpf "
cpcn{ gf "y kj "NE/O U0'Rqukskpu"qh'gzekugf "dcpf u"vqi gvj gt"y kj "eqttgur qpf lpi "tgi kqp"lp"c"vj g'i gn'qh"vj g"xgj keng/"

qpn{ "r wnf qy p"ctg" f gr levgf "kp" Hli 0'570' Cm'r tqvklpu"y kj "j li j guv"ueqtg"cuuki pgf "d{ "RtqvklpRkqv"cpf "cv"ngcuw"6"
f gvevgf "r gr vlf gu'y gtg"i tqwr gf "d{ "eqttgur qpf kpi "dcpf u"qh'v'j g'i gn'y j gtg"v'j g{ "y gtg" f gvevgf 0'Rtqvklpu"tqo "cf lcegpv"
dcpf u"qh'xgj leng"tgcvgf "wo qtu'y gtg"uwdtcevgf "tqo "v'j g"i tqwr "qh'G₄/kpf wegf "r tqvklpu0'0 quv'f qo kpcpv'r tqvklpu"kp"
gcej "dcpf "qh'hli cpf /wko wvvgf "uco r ng"ctg" kpf levgf "kp" Hli 0'570' Vj gtg"ctg"37"o kqej qpf tkcn'r tqvklpu"qww'qh'68"
f gvevgf "r tqvklpu0'I tqwr u"qh'v'j gug"o kqej qpf tkcn'r tqvklpu"XF CEu."CP Vu"cpf "wO vEM"tqto "v'j g"Rgto gcdkklv{ "
Vtcpuksqp"Rqtg"RVR+eqo r ngz"y j lej "tgi wvvgu"gpgti { "hmv"cpf "r nc{ "cp"ko r qtcvpv'tqng"kp"kpklcvkpi "cr qr vaku0'Qv'gt"
r tqvklpu"rkg"o kqej qpf tkcn'Rtqj kdklpu"qt" I CRF J "kpvgtcev'y kj "f hgtgpv'eqo r qpqpwu"qh'v'j g"RVR"eqo r ngz0'Ueqtg-<
Rtqdcdkklv{ "ueqtg."cuuki pgf "d{ "RtqvklpRkqv=" Eqx<Vqcn'eqxgtci g"qh'r tqvklp"y kj "f gvevgf "r gr vlf gu="Ceeguukqp"%<
Ceeguukqp"pwo dgt"qh'eqttgur qpf kpi "r tqvklpu0'kp"dqrf <o kqej qpf tkcn'r tqvklpu0'
"

Hli wt g'58"

"



"

Hli wt g'580" Hwpevkqpcn'cpf "ur cvkn'cuqekvklp"qh'v'j g'r tqvklpu'f gvevgf "kp"v'j g'G₄/kpf wegf "r j qur j qv{ t qulpg"
cuqekvklp "eqo r ngzgu" kp" O EH9<7E" wo qtuo' Vy q" dnwg" rkgpu" tgr tguqpv" qwgt" *QO O + " cpf " kppgt" *KO O + "
o kqej qpf tkcn'o go dtepgu0'Vj g"o clp"eqo r qpqpwu"qh'v'j g'r gto gcdkklv{ "tcpuksqp"r qtg"RVR"ctg"v'j g"XF CE34"cpf "
CP V." y j lej "ctg" cwcej gf "v" QO O " cpf " KO O ." tgr gevkgm{ 0' O kqej qpf tkcn' etgcvlklpg" nklcuq" wO vEM" kp" v'j g"
kpvgto go dtepg'ur ceg'vkuXF CE34"cpf "CP V"qi gv'gt' tqtto kpi "r qtg"eqo r ngz"cu'y gni'cu'r tqxkf kpi "c"rkniv'v'j g"RJ D"
eqo r ngz0'Vj g"RJ D"eqo r ngz"r tqo qvgu'cuugo dn{ "qh'r tqvklp"uwdwku"qh'o kqej qpf tkcn't gur kt cvqt { "ej clp"cpf "qi gv'gt"
y kj "o kqhlkp"ucdkklv{ "etkncg"utvewt g"qh'KO O ." y j lej "eqvklpu"e{ vqej tqo g'e0F wtkpi "cp"cr qr vkle"vli pcn"v'j g"RVR"
r gto gcdkklv{ "kpetgcugu"ngcf "v" quo qvle"uy gmkpi "qh'v'j g"o kqej qpf tkcn'o cvtkz" cpf "tgrgcukpi "qh'v'j g"cr qr vqi gple"
r tqvklpu"tqo "kpvgtetkucn'ur ceg0'Gutqi gp"t gf "uvt+"kpf wegu"tcpuqecvklp"qh'RJ D"r tqvklpu"kvq"pwegwu"dnwg"cttqy + "
tqtto kpi "c"eqo r ngz"y kj "gutqi gp"tgegr vqt"cnr j c"cpf "CKD3"y j gtg"RJ D3"qt"RJ D4"ecp"r tqxkf g"c"tgr tguukqp"hwpevkqp"
qh'gutqi gp/kpf wegf "tcpuetkr vklp"tgi wvklp0'I tggp"rkgp/"pwegt"o go dtepgu0'Vj g"hwmi'rkuv"qh'r tqvklpu'f gvevgf "cpf "
v'j gkt"cetqp{ o u'ctg"tvgf "kp"Vcdng'50'
"

"

Kp"qwt"r tgxkquw"kp"xktq"r tqvgqo leu"uwf { "qh'y kf /v{ r g"O EH9"cpf "O EH9<7E"egmu"*62+."
y g"kf gpwklv{ "CKD3"cu"kpvgtecvkpi "r ctvgt"qh'dqj . 'r tqj kdklp"3"*RJ D3+ "cpf "r tqj kdklp"4"*RJ D3+ "
r tqvklpu." y j lej "ctg"uj qy p"v"dg"r qvgpvcn'tcpuetkr vklp"tgr tguuqtu"qh'GT 0'Rtqj kdklp"4"qt" TGC "
*tgr tguuqt"qh'gutqi gp"tgegr vqt"cevklv{ + "y cu" kpklcm{ "hqwpf "cu"cp" GT "dkpf kpi "r tqvklp"cpf "

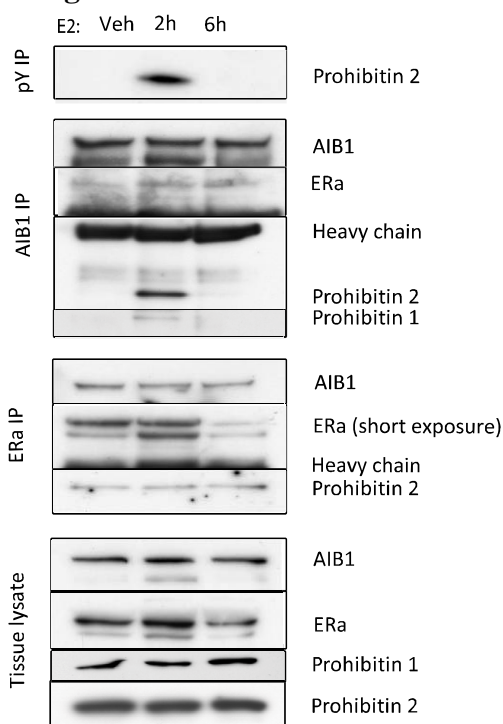
tgr tguuqt0RJ D3'y cu'kf gpvkhgf "cu'c'r qvgpvcn'egm'e { eng'lpj kdkqt'y j lej "dlpf u'r 75.'r Td"cpf "G4HB" r tqvgkpu0"

Kp"qtf gt"vq"eqphko "y j g"O U"hpf kpi u."eqttgur qpf kpi "wo qt"n{ ucvgu'y gtg"uwdlgevfg "vq"cpvk/ r j qur j q/v{ tqulpg"r wmf qy p"hmmy gf "d{ "cpvk/RJ D4"y guvgtp"dmqv"*Hki 0'59+0""Kp"ceeqtf cpeg"y kj " O U"hpf kpi ."y g'f gvevgf "Rtqj kdkk4"qpn{ "4j "chgt "G4"plgevkqp"cpf "k'y cu"cnq"wpf gvevcdrg"chgt" 8j "qh"G40'

Cu"uj qy p"dghqtg."RJ Du"ecp"lpvgtcev'y kj "GT "cpf "RJ D4"*TGC+"ecp"o qf wrcvg"GT " cevxxk{ "d{ "f kuqekcvkpi "C KD3"itqo "tcpuetr vkp"eqo r rgz0*63+"Vq"vj ku"gpf ."y g'r gthqto gf "r wmf qy p"qh"GT "cpf "C KD3"hmmy gf "d{ "y guvgtp"dmqv'y kj "eqttgur qpf kpi "cpvkdqf lgu"ht"GT ". "C KD3." RJ D3." TGC." cpf " XFCE340' " Uwrtkukpi n{ ." y g" hqwpf " dqvj " qh" RJ Du" cpf " XFCE34" eqko o wpqr tgekr kcvgf "y kj "C KD3"lp"zgpqi tch'wo qtu"chgt"4j "qh"G4/vtgcwo gpv0'Vj g"GT "r wmf qy p"tguwv"uwi i guv'y cv'uqo g"qh"RJ Du"eqpukwvkgv{ "cuuqekcvgf "y kj "y j g"GT "r tqvgkpu"*Hki 0'59+0" Vqvcn'ngxgn'qh"cp{ "qh"vj gug"r tqvgkpu"fk "pqv"ej cpi g"dgy ggp"cp{ "vtgcwo gpv'eqpf kkpqu"*Hki 0'59." dqwqo +0"

"

Hki wtg'59"



"

Hki wtg'590Y guvgtp"dmqv'xcnf cvkqp"qh"O U'f cwc0Cpvk/RJ D4"cpvkdqf { "tgeqi pl g"RJ D4"lp"cpvk/r ["R"itqo "wo qt" n{ ucvg"chgt"guvqi gp"vtgcwo gpv"qvr +0RJ D4"cpf "RJ D3"r tqvgkpu"cuuqekcvgf "y kj "C KD3"eqo r rgz" "C KD3"R."qvr lo kf f rg+" qpn{ "lp""wo qtu."j ctxgugf "chgt"4j "qh"guvqi gp"vtgcwo gpv0"RJ D4"ucdn{ "cuuqekcvgf "y kj "tcevkkp"qh"cpvk/GT "r wmf qy p"r tqvgkpu0"Dqwqo <Y guvgtp"dmqv'qh"vqvcn'wo qt"gzvtecv"itqo "wo qtu"qh'xgj lerg."4j "cpf "8j "G4"vtgcwo gpv0'Vqvcn' ngxgn'qh"RJ D'r tqvgkpu"ctg"pqv'tgi wrcvgf "d{ "guvqi gp"cu'lpf kcvgf "lp"y guvgtp"dmqv'qh"vqvcn'wo qt"n{ ucvg0"

"

Cpcn{uku'qhl'paxgnr j qur j qt { r v k p 'u l s u 'q h l o o w p q r w t k l g f 'R J D u 0'

F {pco k" tgetwko gpv' qh' RJ Du" vq" r j qur j qv{tqulpg" eqo r r g z g u" u w i g u u" v j c v" v j k u" k p v g t c e v k p "k u" t g i w r v g f "v j t q w i j "r q u v t c p u r v k p "o q f k l e c v k p "R V O u + c p f "c v" r g c u v" q p g" q h" R J D u" r t q v g k p u" e q p v k p "r j q u r j q v{tqulpg" t g u l f w g u 0' Y g" f g e k f g f "v q" k p v g t t q i c v g" c m" r q v g p v k n" u l s u" q h" r j q u r j q t { r v k p "w u l p i "c" e q o d l p c v k p "q h" r t q v g q o k e u" c r r t q c e j g u 0' k p k l c n" e q p x g p v k p c n" r t q v g q o k e u" c p c n { u k u "N E / O U "y k j "K F C "c n i q t k j o + f k f "p q v t g x g c n" c p { "R V O u "y k j "t g r k c d r g" e q p h k f g p e g 0' W u l p i " w t i g v g f "r t q v g q o k e u" c r r t q c e j "O K F C U / O U + "y g" r g t h q t o g f "c" u w t x g { "q h" c m" r q v g p v k n" r j q u r j q / o q f k l e c v k p "q h" u g t k p g . "v j t g q p k p g" c p f "v { t q u l p g" t g u l f w g u" q h" v q c n r w n f q y p "q h" v j g" R J D 4" r t q v g k p . "v j g" e q k o o w p q r t g e k r k c v g f "R J D 3" c p f "c" h t c e v k p "q h" v j g o "v j c v" e q k o o w p q r t g e k r k c v g f "y k j "v j g" C K D 3" r t q v g k p 0"

Y g" r g t h q t o g f "c" r w n f q y p "q h" R J D 4" c p f "C K D 3" y k j "r t q v g k p" u r g e k l e" c p v d q f k e u" h t q o " w o q t "n { u c v g u" h t q o "c p k o c n u" y k j "x g j k e r g" c p f "4 j "q h" g u t q i g p" t g c v o g p v 0' V j g" c t g c" q h" v j g" i g n' v j c v" e q t t g u r q p f u" v q" v j g" r t g f k e v g f "o q d k k v { "q h" d q v j "R J D u" y c u" g z e k u g f "h t q o "v q r "v q" v j g" d q w q o "k p" u g x g t c n" u r l e g u" c p f "c p c n { | g f "y k j "N E / O U "h q t" r t q v g k p "k f g p v k l e c v k p 0" "C u" y c u" o g p v k p g f "c d q x g . " R J D u" y g t g" h q w p f "e q r t g e k r k c v g f "y k j "C K D 3" q p n { "h t q o "4 j "g u t q i g p / t g c v g f "w o q t "n { u c v g "H k i 0 5 7 + 0' C u" v j g u g" r t q v g k p u" h t q o "u c d r g" j g v g t q f k o g t u . "v j g" R J D 3" r t q v g k p "y c u" e q / k o o w p q r t g e k r k c v g f "y k j " v j g" R J D 4" c p v d q f { "h t q o "d q v j "G 4" c p f "x g j k e r g" t g c v g f "n { u c v g u 0'

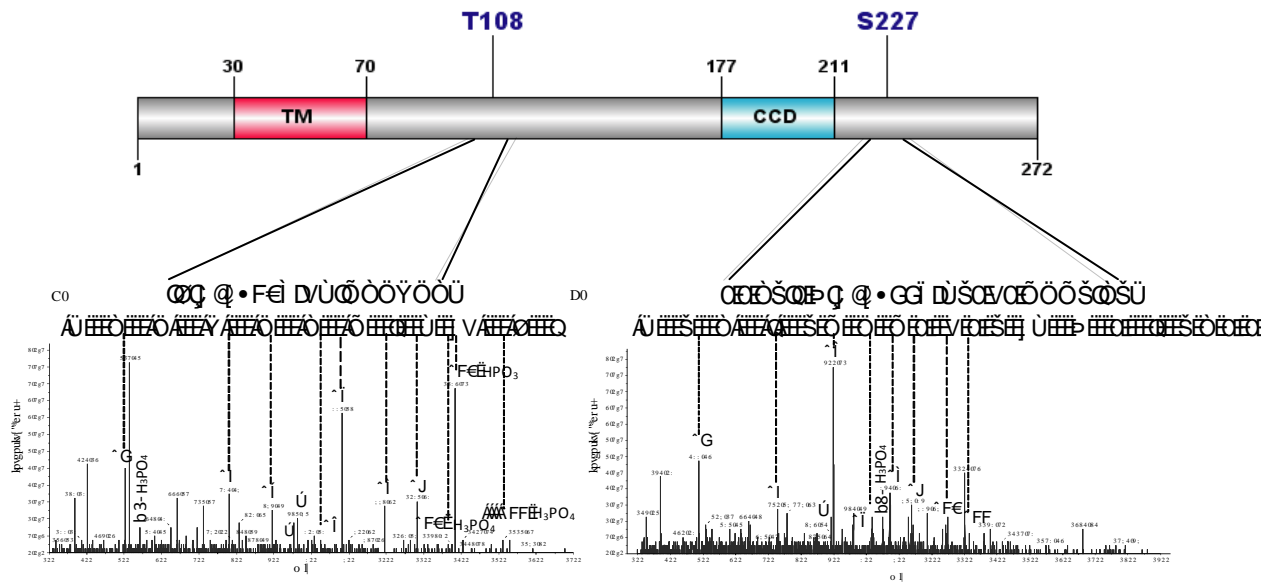
W u l p i "O K F C U / O U . "y g" k f g p v k l g f "v j t g g" p q x g n" R V O u "y k j "j k i j "e q p h k f g p e g" * k p "u e q t g" c d q x g" k f g p v k v { "v j t g u j q r f "w u l p i "O c u e q v" f c v d c u g" u g c t e j "c n i q t k j o + 0' V y q" q h" v j g o . "v j t g q p k p g" c p f " u g t k p g . " e q t t g u r q p f " v q" r q u k k q p u" V j t g / 3 2 : " c p f " U g t / 4 4 9" q h" v j g" R J D 3" r t q v g k p " * H k i 0' 5 : + 0' Y g" f g v g t o k p g f "c" p q x g n r j q u r j q v { t q u l p g" o q f k l e c v k p "q h" R J D 4" c v" r q u k k q p "V { t / 4 9 4 * H k i 0 5 : + 0' "

"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"
"

Hl wt g'5: "

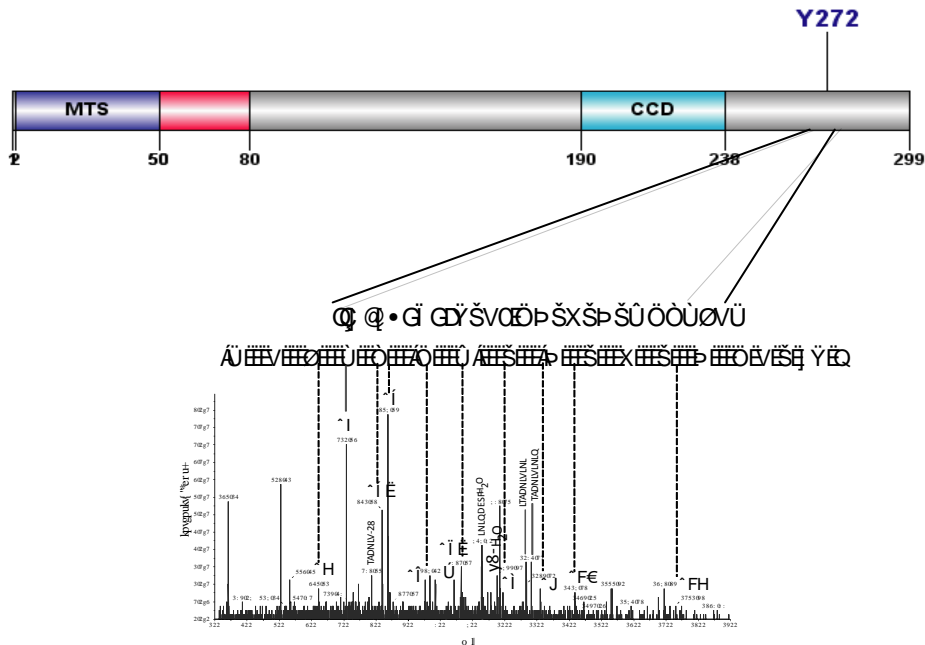
"

ÁÚÓ



ŒÈ

ÁÚÓÓ



Hl wt g'5: 0Uej go c'v'e'f qo clp'lt wewt g'qh' RJ Du' C/' RJ D. 'c'pf 'D/' RJ D4+'y kj 'r qukskpu'qh'paxgnt j qur j quksu' c'pf 'eqttgur qpf lpi 'c'pf go 'O U'lt ci o gpw'clp'ur gev't c0'Uej go g'ngi gpf <O VU/'o kqej qpf tlc'v'ti g'kpi 'ugs w'peg." VO/''t'cpuo go d'tcpg'f qo clp. "EEF/'"eqk'eqk'g'f 'f qo clp0' RJ D' *qt' URHJ '+'f qo clp'u'eqttgur qpf u' 'q' ugs w'peg' 'tgi k'p' dgvy ggp' 'r qukskpu'48/3: 9' c'pf '5; /423' co l'p'q' c'ekf 't'guf w'gu'qh' RJ D3' c'pf ' RJ D4' t'gur g'v'x'g'n 0' V'c'pf go 'O U'ur g'v'c' 'qh' r j qur j qt { r'v'g'f 'r gr w'f gu'y kj "d/v' r g' c'pf " { /v' r g' k'pu. "l'p'w'f lpi "p'g'w't'c'n'quu' k'pu'qh' r j qur j q't'le' c'ekf "l'p' r quks'x'g' k'p"

o qf g"qh"O UIO U" wulpi "Eqmukqp" kpf wegf "F kuqekvqp" ctg" kpf lecvgf "cu" / J 5RQ6_ "hqt" pgwtcn" nquu" qh"; : "F c." cpf " / " J RQ5_ " hqt" pgwtcn" nquu" qh" : 2" F c0" Vj gug" ej ctcevgtkvle" kpuu" y gtg" qdugt xgf" qpnf" hqt" r gr vlf gu" eqpcklpki " r j qur j qt { r vgf " ugtkpg" cpf " yj tgpklpg0 kpf cf f kklqp " vq " pgwtcn" nquu" qh" r j qur j qtkle " cekl . " uqo g" qh" yj g" r tqf wev" kpuu" ncdgngf " kp" yj ku" ur gestwo " j cf " o cuugu" yj cv" y gtg" 39" F c" cpf " 3: " F c" nqy gt " yj cp" yj g" cewcn" o cuugu" f vq" yj g" pgwtcn" nquu" qh" P J 5" cpf " J 4 Q" t gur gevkgngf 0 Vj g" r tqf wev" kpuu" r gcmu" o quvnf " { " ugtkpu" cpf " uqo g" qh" d" ugtkpu" yj cv" y gtg" o quv" f kci pquke" qh" yj g" r j qur j qt { r vgf " ukgu" ctg" kpf lecvgf " cnqpi " y kj " yj g" ugs wvpeg" qh" yj g" r gr vlf g0 C0 Ngn" ur gestwo < Vcpf go " O U" ur gestwo " qh" kfr j qu32: + VUK GF [F GT " r gr vlf g" eqttgur qpf kpi " vq" r quklqp " 328/339" cc" qh" RJ D3 " r tqvklp " r tgewtuqt " kqp" y kj " o / " 9440 " cpf " ej cti g" ucvg"] - 4J _4- +0" EKF " Hci o gpvcvqp" r tqf wegf " pgwtcn" nquu" *; : " F c+" qp" r j qur j qt { r vgf " yj tgpklpg" t gukf wgu0 U" ur gestwo " j cxg" utqpi " { " ugtkpu" kpuu" y kj " pq" P N" vr " vq" { ; " kpf lecvqp" yj cv" pq" r j qur j qt { r vgf " qp" U" cpf " [" co kpg" cekl u0 P Nu" qp" d5 . " { 32" cpf " { 33" uwi i guv" r j qur j qt { r vgf " qp" V32: 0 Vj g" f cecdug" ugcetj " y kj " O cueqv" cni qtkj o " i kxgu" c" ueqtg" qh" 77 " c" ueqtg" cdq xg" 49" kpf lecvgu" kf gpvksf +0 T ki j v" ur gestwo < EKF " Hci o gpvcvqp" ur gestwo " qh" CCGNKCP * r j qu449 + UNC VCI F I NIGNT " v { r vlf g" qh" RJ D3 " r tqvklp " cvr quklqp " 442/45; " cc" r tgewtuqt " kqp" y kj " o / " 8; 506 =] - 5J _5- +0" O cueqv " 64 " * @ 9 " kpf lecvgu" kf gpvksf +0 " Vj gtg" ctg" pq" pgwtcn" nquu" *; : " F c+" qp" kpuu" { 32" cpf " { 33" eqpcklpki " vpr j qur j qt { r vgf " yj tgpklpg . " y j gtgcu"] d: / J 5RQ6_ " kqp" o cr r gf " r quklqp" qh" o qf kkecvqp" vq" U4490" D0 O UIO U" ur gestwo " qh" r tgewtuqt " kqp" cv" o / " 98; " qh" vkr ng" ej cti gf " vkr vlf " r gr vlf g" kfr j qu494 + NVCF P NXNP NS F GUHVT " qh" RJ D4 " r tqvklp " cvr quklqp " 494/4; : 0" O cueqv" ueqtg " 56 " * @ 9 " kpf lecvgu" kf gpvksf +0 U" utqpi " { " kqp" ugtkpu" * y kj " vngtcepg" vr " vq" 208: " F c+" grko kpcvgu" co dki vksku" y kj " r j qur j qt { r vgf " qp" r quklqp " U4: 8" cpf " V4: : 0 Vj gtg" ctg" o vej " ngu" d / " kpuu . " r tqdcdnf " f vq" vq" kpgt pcn" Hci o gpvcvqp 0 Vj g" d { / kpuu" qh" vpr j qur j qt { r vgf " kpgt pcn" Hci o gpv " kmg " NVCF P NXNP " cpf " VCF P NXNP S + grko kpcvg " c" r quklklksf " qh" r j qur j q / " o qlkv " qp" V496" cpf " r qlpvqy ctf " r j qur j qt { r vgf " qp" [4940 " " "

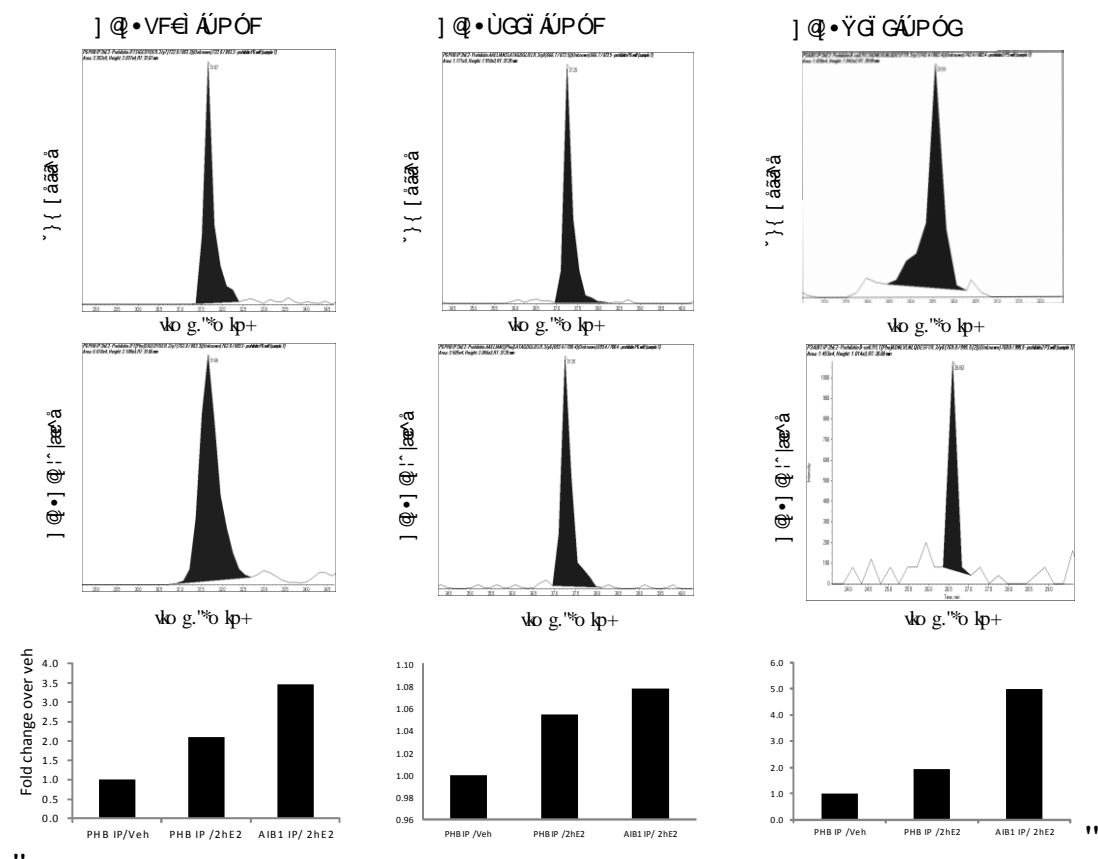
F gvgto kpcvqp" qh' r j qur j qt { r vgf " uvklej kqo gvt { " qh' pqxgn' gvtqi gp / kpf wegf " r j qur j qv gr vlf gu0 "

Vq" i cklp" o qtg" wpf gtucpf kpi " qh" j qy " yj gug" r j qur j qt { r vgf " ukgu" ctg" tgi wrcvgf " d { " gvtqi gp . " y g" wugf " O TO / O U" deugf " s wcpvkvqp 0 Vj g" rkuv" qh" vcpuklqp " wugf " hqt " s wcpvkvqp " cpf " vki i gtlpi " O UIO U" ku" r tguvpgf " kp" Hki 0 5; +0" Rgcmu" y gtg" kpgi tcvgf " wulpi " s wcpvkvqp " uqhy ctg" O cnkS wcpv" 408 " * CD " Uelgz . " Hico kpi j co . " O C +0" Cp " cdwvf cpeg" qh" r j qur j qt { r vgf " r gr vlf gu" ku" f gvgto kpgf " Htqo " yj g" uij cr g" qh" yj gk " Hci o gpv" r gcmu" kp" eqttgur qpf kpi " O TO " wulpi " yj g" ctgc" wpf gt " yj g" ewt xg" vq" i gpgtcvgf " yj g" uki pcn" tgrvkvxg" vq" yj g" tgrvvgf . " wpo qf kklgf " r gr vlf g" * 64 . " 65 +0 Cu" cp" cf f kklqp cn" eqpvtn " y g" wugf " v { r vlf g" r gr vlf gu" Htqo " yj g" uco g" r tqvklp " cu" cp" kpgt pcn" ucpf ctf 0 Vj ku" o gvj qf " j cu" dggp " f guetkdgf " cu" yj g" opcvkvxg" tghgtgpeg" r gr vlf g0 " * 65 +0 Vj g" kpgt pcn" ucpf ctf " r gr vlf g" eqpcklpkf " pq" r qvvpvkmf " o qf kklgf " t gukf wgu" cu" c " tguwn " qh" RVO u" qt " uco r ng" r tgr ctcvqp " o qf kkecvqp " uvej " cu" e { uvklpgu" cpf " o gvj kplkpgu" cpf " pq" kpf lecvqp " qh" o kuugf " engcxi g0 Vj g" wug" qh" kpgt pcn" ucpf ctf u" * tgrvvgf " wpo qf kklgf " r gr vlf g . " qt " opcvkvxg" tghgtgpeg" r gr vlf g0 + ku" tgs vkt gf " vq" eqo r gpucvg " hqt " c " f khtgpeg " f vq" xctkvqp " kp" kpr w0 o cvgtkn " tgeq xgt { " qt " kputwo gpv" t gur qpug0 F cwc" qh" rki cpf / uko wrcvgf " uki pcnu" y gtg" eqo r ctgf " vq" eqttgur qpf kpi " uki pcnu" Htqo " cp" wpuvko wrcvgf " gzr gtlo gpv" cpf " r tguvpgf " kp" yj g" i tcr j " cu" hqrf u" qp" kpf wevqp0 "

Y g" hqwpf " yj cv" y q" pqxgn" RVO u" ctg" gvtqi gp" kpf wekdng " cv" ngcu" vy q / hqrf . " cu" eqo r ctgf " vq" xgj kerg / qpnf " wo qtu +0 Y g" qdugt xgf " gptlej o gpv" qh" r j qur j q / RVO u" kp" Htcecvqp " qh" RJ Du" cuuqekvgf " y kj " gvtqi gp" kpf wegf " C KD3 " eqo r ngz " * Hki 0 5; +0" Gur gekmf " utknkpi " ku" yj g" cm quv" 5 " vko gu" rti gt " tgetvko gpv" qh" r j qu / V { t / 494 " RJ D4 " vq" yj g" C KD3 " eqo r ngz " xgtuwu" yj g" ngxgn" qh" gvtqi gp / uko wrcvgf " r j qur j qv { tqulpg " Htcecvqp " qh" vqcn" RJ D4 " * Hki 0 5; +0" C v" yj g" uco g" vko g . " y g" qdugt xgf " y leg" o qtg" r j qu / V { t / 494 " kp" G4 / vgcvgf " wo qt " yj cp" kp" yj g" xgj kerg / qpnf " RJ D30 " "

Hli wt g'5; "

Oqf kh	Rtqgkp	Rgr wf g	Ejcti g lqkp	S3	S5
r V32:	RJ D8	K NVJRj q_CFPNKNPNSFGUHT	5 l(9	96406	" : : 406
	RJ D8	K NCFPNKNPNSFGUHT	5 l(9	9446	:: 406
r U49	RJ D8	CCGNKP JRj q_UNCVCI FI NGNT	5 l(8	8; 506	92206
	RJ D8	CCGNKP UNCVCI FI NGNT	5 l(:	88809	: 9407
r l 494	RJ D4	KJRj q_ NCFPNKNPNSFGUHT	5 l(:	98;	:: 707
	RJ D4	K NCFPNKNPNSFGUHT	5 l(9	96406	:: 406



Hli wt g'5; 0'S wcpwskvqp'tpcnf uku'qhgut qi gp'lpf wegf 'r j qur j qo qf kkecvkp'wulpi 'O TO /O U'

C0'O TO "tcpukskpu"ht"O TO /O U"cpnf uku'O qf k0/"r qukskqp"qh'r j qur j qt {nkvqp"kp"eqttgur qpf lpi "r tqvklpu"y kj "tgur gev'q"r tqvklp"cpf "r gr wf g"ugs wpeg0"S 3"cpf "S 5"/o l "ugwkp u"ht"s wcf tw rgru"lp""S VCR6222"kpwtwo gpv'ht"tgi kntcvkp"qh'gcej "rtgewtuqt lhtci o gpv'r cku0'Ej cti g/"r tqvklpcevq "ucvq"qh'r tgewtuqtu."htci o gpv'"/v'r g"*ectdqz {n"htci o gpv'f gvevqf "lp"j g'O TO 0'

D0Vqr "tqy /"gztcvqf "kqp"ej tqo cvqi tco u"*Z K+"qh'tgr tguqpcvkg'O TO u"qh'r j qur j qt {nkvqf "r gr wf gu"wgqf "ht"j g"s wcpwskvqp"kp"j ku"uwf {0'F cvc"y gtg"pqto crk gf "vq"eqttgur qpf lpi "wpo qf kkegf "r gr wf gu"*Z K+u"qh'O TO u"lp"j g"ugeqpf "tqy 0'P qto crk cvkp"y cu"xcrkf cvqf "wulpi "j g"kpvtpcn'r gr wf g"htqo "j g"eqttgur qpf lpi "r tqvklp"cu"cp"kpvtpcn'ucpf ctf 0'

Rt qvglp'hkpcug'r tgf levkqp'qh'j qur j qt { rlvkqp'hlsgu'd { 'o qvhlhwdutcvg'tecppkpi 'r tqi tco u0'

Ko rqtcpv' i qcnu' hqt" vj g" hwwtg" ctg" vq" kf gpvkh{ " vj g" r tqvklp" nkpcugu" tgr qpukdrg" hqt" r j qur j qt { rlvkpi "qh' RJ D" r tqvklpu" kp" xkxq" cpf" vq" f gvgto kpg" j qy " r j qur j qt { rlvkqp" cv" gcej " uksg" chgeu" vj g" hwpevkqpcn' tgr qpug" vq" nki cpf" vtgcvo gpv0Vq" f gvgto kpg" y j lej " r qvgpvkcn' r tqvklp" nkpcugu" eqwrf " dg" tgr qpukdrg" hqt" r j qur j qt { rlvkqp" qh' vj g" kf gpvkhkf " r j qur j qulsgu. " y g" wugf " vj g" uwdutcvg" r tgf levkqp" r tqi tco " Rj qur j qO qvkh/Hkpf gt" [j wr <1y y y Œ r tf Œti Rj qur j qO qvkh/hkpf gt](#) " *66-0' Vj g" ugs wgegu" qh' RJ D" r tqvklpu" y gtg" wugf " vq" kf gpvkh{ " r tgf levkf " nkpcugu" vj cv" ecp" r qvgpvkcn{ " r j qur j qt { rlvg" RJ D" r tqvklpu0' Vj g" Rj qur j qO qvkh/Hkpf gt" r tgf levkf " vj cv" Vtg/32: " eqwrf " dg" r j qur j qt { rlvf " "" d { " " EJ MB. " RME " "" qt " d { " hco kn{ " qh' ecrkwo lecm qf wkp " EcO +f gr gpf gpv' r tqvklp " nkpcugu" * " EcO MKK qt " EcO MKK -0' Vj g" r tgf levkqp" hqt " Ugt/449" ctg" I " r tqvklp/eqw rnf " tgegr vqt " nkpcug" 3. " Ecuglp' Mpcug' Kcpf " KK" ugg' uwdutcvg" r tgf levkqp" kp" Vcdrg" 6-0' Vj g' emuguv' nkpcug" o qvkh" vq" V{ t/494" qh' RJ D4 " ku" Z } F IG _ r [] K NIX _ " hqt " GI HT 0 " Qv gt " y gd " tguqwtg " wkrk kpi " ct vhlkcn' pgwtcn' pgwy qtn' dcuqf " r tgf levkqp" vj g" P gvRj quM' *67+ " *j wr <1y y y Œ luf wff mltgxlegulP gvRj quM+ " cuuki pu" V{ t/494" vq" GI HT " v{ tqukpg' nkpcug" y kj " c" r tqdcdkkrk{ " qh' 20' *? " 72' -0' "

Vcdrg'6''

"

r j quVtg/329' RJ D''

325"/"32: ""	NRT KHV""	JO IKNIX_ZJTIMZZJrUlrV_""	Ej nŒ' nkpcug' uwdutcvg' o qvkh''
325"/"32: ""	NRT KHV""	JO IKNIX IHI[_ZTZZJrUlrV_""	Ecm qf wkp/f gr gpf gpv' r tqvklp' nkpcug' KK' uwdutcvg' o qvkh''
325"/"332""	NRT KHVUK'	JO IX NIKH ZJTIMZZJrUlrV_ZZ""	Ecm qf wkp/f gr gpf gpv' r tqvklp' nkpcug' KK' uwdutcvg' o qvkh''
327"/"32: ""	T KHV""	TZZJrUlrV_""	Ecm qf wkp/f gr gpf gpv' r tqvklp' nkpcug' KK' uwdutcvg' o qvkh''
327"/"32: ""	T KHV""	JTIMZZJrUlrV_""	RME' nkpcug' uwdutcvg' o qvkh''

"

Rj qu/Ugt/449' RJ D''

448"/"453""	P UNVCV""	ZJrUlrV_ZZZJCIRUIV_""	I " r tqvklp/eqw rnf " tgegr vqt " nkpcug" 3' uwdutcvg' o qvkh''
449"/"452""	UNCV""	JrUlrV_ZZZJUIV_""	Ecuglp' Mpcug' K' uwdutcvg' o qvkh''
449"/"452""	UNCV""	r UZZJGlrU, lrV, _""	Ecuglp' Mpcug' KK' uwdutcvg' o qvkh''

"

Vcdrg'60' Ego r wewkpcn' r tgf levkqp' qh' c' nkpcug' o qvkhgu' qt' uwdutcvgu' hqt' pqxgn' r j qur j qulsgu' *f gvcku' kp' vj g' vgzv+ "

Eqpenwukpu'

Cp" cpcn{ uku' qh' o TP C" gzt tguukqp' tgi wlvkqp" chgt "6: " j tu' qh' gutf kqn' tgcvo gpv' qh' O EH/9" r ctgpcn' cpf " O EH/9 <7E" egmu' y cu' r wdrkuj gf " tgegpvn{ " *, +d { " vj g' EQG" i tqwr " cpf " u j qy u' uki pklkcpv' f khtgpegu" y kj " tgr gev" vq" o TP C" gzt tguukqp" qh' tgi wlvqtu' qh' cr qr vuku' cv' ugcf { " uvcg" *68- < " kŒ " O EH/9" egmu. " Den/4. " c" o clqt " cpk/ cr qr vuku" i gpg. " ku" w' tgi wlvf " d { " gutf kqn' tgcvo gpv' y j gtgcu" pq' ej cpi g' qh' Den/4' y cu' uggp' kp' O EH/9 <7E" egmu0'

Qxgtcm' vj g' gzt tguukqp' cpcn{ uku' cpf " r tqvgqo leu' f cv" u j qy " uqo g" lpygtgukpi " eqpxgti gpegu" gur gekm{ " kp" cr qr vuke" tgi wlvqt { " r vj y c { u' y j lej " o c { " dg" hwpevkqpcn{ " tgrgxcpv' cu' lpkkcvqtu' qh' gutf kqrókp f wegf " cr qr vuku0'

Rtgxkqwn{ . " y g' ej ctcevgtk gf " O EH9 <7E" egmu' kp" xkt q" d { " c" r tqvgqo leu' cpf " lphqto cvku" cr r tqcej . " hpf kpi " uki pklkcpv' r vj y c { " cngtcvku" htqo " vj g" r ctgpcn' egm' nkpg" chgt" gutfi gp"

unko wrwvkqp" *62-0" K'j cu" dggp" uwi i guvgf "htqo "cp" kp" xktq" uwwf { "y cv" o kqej qpf tkc" j cxg" dggp" uki phtecpwnf "kpxqkxgf "kp" kpkkcvkp" qh" cr qr vquku" kp" y g" OEH97E" egm0Y g" hqwpf "RJ D3" cu" qpg" qh" y g" kpvgtcevpki " rctvpgtu" qh" C KD3" eqcevwxcvt0 Vq" hwt y gt" gzt rqtg" y gug" hkp f kpi u. " y g" wugf " OEH97E" wo qt" egm1 kp" c" zgpqi tchv" o qf gnl vq" gzvgpf " qwt" npqy rnf i g" qh" gwtqi gp/ kpf wegf " cr qr vquku" kp" xkxq0"

Hwpevkpcn's wcrk{ 'c'pf 'uli p'k'ec'peg'q'h'c'p'k'p'gt'cev'k'p'q'h'RJ Du'y k'j 'v'j g'k't'r'c't'v'p'gt'u0"

Upeq'o quv'qh'j g'y qtnl'kp'j g'r wdrkuj gf "rksgtcwtg'y cu'r gthqto gf "wpf gt's wksq'ctwklecln' eqpfkklqpu'rkng"qxgtgZR tguukqpu'qh'RJ Du." {gcuv/y q"j { dtkf "u{wgo "gve."qpg'qh'j g"cti wo gpw' eqwff "dg'tckugf "cdqw'suvenf' ej cr gtqpu'tcvj gt'j g'ur gekkle'kpvgtcevkqp'y kj "r ctvpgtu"*69+0Y g' uj qy "j cv.'cv'ngcu'kp'qwt'o qf gn'RJ Du'kpvgtcevkqp'y kj "uki pcnr'tqvkpu'ku'f { pco ke."tgi wrcvgf 'd {" RVO u.'cpf "vo g/f gr gpf gf 0Vj gug'tguwmu'wrr qtv'ur gekklek'qh'hqwpf 'kpvgtcevkqpu'0"

Hqt"vj g"cdqxcg'o gpxkqpgf"tgcuppu."qwt"pgy"fcw"uj gf"rki j v'qp"vj g'r tglxkwu'hpf kpi u0Kj"cu" dggp"f guetldgf"vj cv'lp"dtgcu'ecpegt"RJ D3"kpwtcev'y kj"O NM4"%o kz gf"nkpgci g'nkpcug"4."dgmipi u" vq"O CR5M'encuu'qh'nkpcugu'cpf"tgi wrcv'u'r 5: ILP M/uki pcrkpi"rcvj y c{-0Qp"vj g'qy gt"j cpf."RJ D3" y cu"ko r necvgf"kp"tgi wrcv'qp"qp"TCU/TCH"uki pcrkpi"6: ."6; -0I kxgp"vj g"tgr qtvgf"Ugt IVj tg" r j qur j qt {rcv'qp"ukgu"qp"RJ D3."kv"y qwf"dg"vgo r kpi"vq"cuuguu'hwpv'kqpcn'tgrgxcpeg"qh"vj ku" kpwtcev'qp'y kj"tgi ctf"vq"vj g'f guetldgf"r j qur j qt {rcv'kpu0

Dqy " r tqvku" hto " c" nti g" tpi /rkg" eqo r ngz." cpej qtgf " v" y g" kpgt" o kqej qpf tkcn' o go dtepg'y j gtg'y g' hwekq"cu'ej cr gtqpgu."uwr r qtv'o kqej qpf tkcn'kpgi tk' "cpf 'r j { ukmji { 0K' y cu" pqlgf " y cv' grxcvgf " ngxgn' qh' y g" RJ D" r tqvku" qhgp" eqttgrvg" y kj " o kqej qpf tkcn' f { uhwekq"cpf "eqwf "dg"wugf "cu'o ctngtu"qh'ko dncpeg"qh'y g'tgur kcvqt { "ej clp"*69-0F wg"v" y g" eqo r ctvo gpwnk cvkq"ghgev."y g"hwekq"qh'y gug"r tqvku"y qwf "dg"gj gt"r tq/i tqy y lwtlxcn' qt"r tq/cr qr vkle lpgi cvkg'tgi wrcvtu"qh'egm'e { enp0"

K'y cu'r tqr qugf "y cv'cp'lpetgcugf "rgxgn'qh'RJ D'r tqvgkpu'ku'c'f ghgpug'o gej cpkuo "y cv'y g" egn' vtgu' vq" ko r necvg" kp" tgr qpug" vq" o kudcrpegf " r tqvgkcp0' " Vj ku" kf gc" ukm' tgs vktgu' gzt gto gpvri'xcrkf cvkqp0'O kqej qpf tlen'r tqj kdkkpu'kpvtcev'y kj "CP Vu'cpf "XF CEu'r tqvgkpu'cpf " ucdk' g'y g"Rgto gcdk' "Vtcpu'kqp"Rqtg"*RVR+"eqo r rgz0'Vj g"RVR"tgi wrgu'gpgti { "huz"cpf " r r { u'cp'ko r qtcvptqg'kp'kpkcvpi "cr qr vuku0'

QUV6: 'r t q v k p ' c p f ' F C F 3 ' * v j g ' f g h p f g t ' c i c k p u ' e r q r v w k e ' e g m f g c v j ' 3 + '

Vj gtg"ku"cpqj gt"kpvtgukpi "nkm'dgwy ggp"yj g"QUV6: "rtqvgkp"cpf "FCF3"*yj g'f ghpf gt" ci ckpuv' crqr vqle" egm' fgcjy" 3-0' Dqjy" rtqvgkpu" ctg" dgrkxgf" vq" dg" uwdwpku" qh' yj g" qrk quceej ct { ntcpuhgtcug" eqo r ngz0' Vj g" mqu" qh' FCF3" hpxevkp" kpf wegu" crqr vquku0' Vj g" yq/ j { dtkf "u{uvgu u'lpqxrkpi "FCF3"cu'dck'r klpw"vqy ctf "yj g'O en/3'rtqvgkp."apg"qh'yj g'Den4'ho kn."

O kqej qpf tkn'čpf 'b gvcdqne/'t gi wv'gf 'r t qv'kpu''

I CRF J "y cu"tgr qtvgf "vq"ceewo wrcvg"lp"o kqej qpf tlc"fwtkpi "cr qr vquku."cpf"lpf wegu"y g" r tq/cr qr vqk"o kqej qpf tlc"n"o go dtepg"r gto gcdkkl cvkqp."vki i gt kpi " y g"lpvtkpule"r cvj y c{" qh" cr qr vquku"*/3+0Vj ku'r cr gt"tgr qtvgf "cp"cuuqekcvkqp"qh'I CRF J "y kj "XF CE"ego r rnz0'

"

Et quæcūq; ggp'ò kqej qpf tk'çpf 'gpf qrxuo cyle't gylewwo '*GT+'

Vj g"ugeqpf "i tqwr "qh'r tqvklpu" f gvevzf "kp" qwt "o cuu'ur gevto go gt { "cpcn{ uku'qt ki kpcvzf "ltqo " vj g" gpf qr nuoo ckle "tgvevno " *GT +0" Vj g" GT "r r { u'cp "ko r qtcvpv' tqv' "kp" o ckvcklpi "vj g" e { vquqrlc " rxxgn'qh" ecrekwo " *74+ " cpf " tgi wrcvki " vj g" cr qr vqve "t gur qpug" qh" o kqej qpf tlc0' kpgt guvki n{ . " qpg" GT " cuuqekcvzf " r tqvklp " Gtrkp / 4 " *GT " rkr kf " tch' r tqvklp + " j cu " c " ugs wpeg " uko krtkl { " y kj " r tqj kdklpu " cpf " hcm' y kj kp " vj g" i tqy kpi " hco kn { " qh' r tqj kdklpu " f qo ckv / eqpvcklpi " *RJ D+ " r tqvklpu. " y j lej " kpenmf gu'vj g' r tqj kdklpu. " vj g' uqo cvku " cpf " vj g' hqvklpu " *75. " 76+0 " "

Ctlc| k'gv'cn'eqpf wevzf "i gpg" gzt tguvqp " uwf { " qh " gvtqi gp " kpf wegf " cr qr vquku. " eqo r ctkpi " OEH9 " egm' xctkcpw " vj cv' y gtg " gvtqi gp / f gr gpf gpv " *Y U: + " qt " tgukvcpv " vq " gvtqi gp " f gr tkxcvklp " *4C+ " qt " ugpuvklxg " *7E+ " vq " G4 / uko wrcvzf " cr qr vquku0 " Vj g { " hqvzf " uki pkkecpv " cuuqekcvzf " cpf " qxgttgr tguvckvqp " qh " gpf qr nuoo k " tgvevno " utgu " *GTU+ " i gpgu " co qpi " vj g " 7E / ur gekhe " i gpgu0 " Vj ku' rkpnlkf kecvzf " vj cv' G4 + kj kdkvzf " r tqvklp " hqv kpi " cpf " vtcvurckvqp " *, +0 " "

Hwpevklpcn' t gixcpeg' qh' bpxgltgvt qi gp / kpf wegf 'RVO u' qh' RJ Du' t qvklpu0'

F gur kg " vj g " i tqy kpi " dqf { " qh " gxlk f ppeg " rkpnlpi " RJ Du " vq " vj g " gvtqi gp / f gr gpf gpv' r cvj y c { . " rkwg' ku' npqy p' cdqwtgi wrcvqp " qh' vj gug' r tqvklpu " d { " r j qur j qt { rkvqp " kp " vj ku' eqpvz v0Gxqnvklpct { " eqpugtxcvklxg " cpf " r j { rqi gpgvecm { " xgt { " cpekvpr' r tqvklpu " uwi i guv' vki j v' hwpevklpcn' tgi wrcvqp. " cu " y gni' cu " d { " RVO u0 " Hqt " gzc o r ng. " RJ D " eqpvcklpu " qpn { " 6 " v { tqvklp " tguv wgu' eqpugt xgf " cetquu' tgo qvg " ur gekgu " *ltqo " j wo cp " vq " f tqvkr j krc+ " cpf " gxgp " uqo g " j cxg " j qo qm { " kp " RJ D40 " Rtqdcn { " cni' qh " vj go " ctg " tgi wrcvzf " d { " r j qur j qt { rkvqp " *77. " 78+0RJ qur j qt { rkvqp " qh' RJ D " cvV { t / 336 " cpf " V { t / 47; " y cu' f guetklkf " vq " dg' tgi wrcvzf " d { " kpuvklp lkt " *77. " 78+ " y j gtgcu " Vj tg / 47: " ku' tgi wrcvzf " d { " Cm " *79+0 " Rj qur j qur gekhe " f cvdcugu " eqpvckl " lphqto cvklp " cdqwt' cf f kkvpcn' RVO u " qh' RJ Du " y j lej " eqo g " ltqo " r tqvgo ku' r tqvklpi " cpf " uknltgs vktg' xcrlf cvklp " cpf " cuuguoo gpv' qh' hwpevklpcn' t gixcpeg0 " "

Vj g " o clqt " tguvckvqp " hqt " uwf { " cpf " gxcvrcvklp " qh " ppxgn' r j qur j qt { rkvqp " ukgu " ku " vj g " cxckrdklk { " qh " ko o wpqej go kecn' tgei gpv. " uvej " cu " r j qur j qur gekhe " cpvklkf lgu " hqt " Y guvtp " dmrvkpi 0Vq " qxgteqo g " vj ku' rko kecvqp. " y g " ko r ngo gpvzf " cp " cuuc { " qp " vj g " dcug " qh' vj g " NE OTO / OUr rkvqto " vq " gpcdrf " vj g " o gcwtgo gpv' o gvj qf0 " "

Vq " vj g " dguv " qh " qwt " npqy ngf i g. " f gur kg " c " pwo dgt " qh " r wdrckvklpu " rkpnlpi " RJ Du " vq " vj g " gvtqi gp / tgi wrcvzf " r cvj y c { . " vj gtg " ctg " pq " f cv " f guetklkpi " j qy " RJ Du " ctg " tgi wrcvzf " d { " r quv' vtcvurckvpcn' o qf kkecvklpu0 " "

"
"

VCUM6 < *HEEE lCtcl k' VI gp lEwpnlhg = Lqtf cp lI W' d' Vq' cpcn { | g' G4 / kpf wegf ' lwt xlcrcn' cpf " cr qr vqve' r cvj y c { u' wklpi ' l gpg' cttc { u' cpf ' lktP Cu0'

Vcunl6c < *Ewpnlhg. C | qtuc. ' Dcni wt wpcvj cp + / " kpgt tqi cvg' r cvj y c { u' qh' gpf qetkpg' t gukvcpv " wklpi ' j ki j ' vj tqwi j r w' TPC " kpgt lgt gpeg " *J V / TPC k "

kpvtf wevklp < "

J gtg " y g " tgr qtv " y qtnl' eqo r ngvzf " qp " Vcunl' 6e " cv " Vj g " Vtcvurckvpcn' l gpqo ku " Tgugetej " kpuvkwg " *VI gp + " ukv " f wtkpi " { gct " 70 " Y g " tgr qtv " cp " kp " f gr vj " cpcn { uku " qh " vj g " wy q " tgr rkecv " j ki j " vj tqwi j r w' TPC k " Ftwi i cdng " l gpqo g " uetggpu " tguvklpi " kp " c " r tkqtkk gf " rkuv " qh " 499 " i gpgu " *ltqo " 9222 " uetggpgf + " y j qug " kpj kdklqp " ghgvevkn { " dmqem " G4 / kpf wegf " cr qr vquku " kp " vj g " OEH9 < 7E " egm' rkg " o qf gr0 k r qtcvprn { . " vj gug " 499 " i gpgu " y gtg " ugrgevzf " pqv' qpn { " f wg " vq " vj gk " hwpevklpcn { "

r tgf levgf "r tqvgevkxg" ghgevg'qh'G₄/o gf kvvgf "cr qr vuku"lp"O EH9-7E."dw"vj g{"y gtg"cf f kkpccm{"pqv"
e{"vqvzle"vq"O EH9-7E"egm"lp"vj g"cdugpeg"qh'G₄"i gpg"vcpuetkr w"y j qug"lpj kdkkqp"ku'e{"vqvzle"
y kj "qt'y kj qw'c'r j cto ceqrqi kecn'ci gpv'tg'v'r kecm' tghgtt'gf "vq'cu'Cej kngs' hegn'vcti gwu-0'"

C"eqo r tgf gpukxg"gzr rncvkkp"qh'vj g"o cvj go c'kecn'cr r tqcej "vq"r tkqtkk'g"vj g"ecpf kf cvg"
rkuv' qh' šG₄/rtqvgevkxg' i gpgu" y cu" r tqxkf gf "lp" vj g" [gct" 6" hkp'cn' tgr qtv' *F t0' [qi cpcpf "
Derni wtwpvj cp-0' Dtlghn'." f cvc" y gtg" cpcn' [gf "lp" c" r ncvg/y kug" hqto cv' wulpi "c" xctkv'{"qh"
pqto crk' cvkqp" r tqegf wtgu" eqo o qpn' "wugf "lp" r wdrkuj gf "rkgtcwtg" hqt" TP CK' uetggp'lpj "f cvc0'
O gj qf u"lp'ncf gf <o gf kcp"pqto crk' cvkqp."o gf kcp/r qrkuj ."D/ueqtg."Hktuv' qtf gt" cpf "ugeqpf "qtf gt"
tgi tguukqp0Hqt'c" f gckxgf "f guetkr vkp"qh'o gj qf u'wugf ."r ncug'tghgt'vq'cr r gpf gf "f qewo gpv'd{"F t0'
Derni wtwpvj cp"*šC"r tqeguu"o qf gn'vq"gxncv'g"j k'tcv'g" f kueqxtg{"ö"r tgu'pvgf "cpf "r wdrkuj gf "lp"
cuuqek'cvkqp" y kj " vj g" 422: " KGG" I gpqo leu" Uki p'cn' r tqeguukpi " cpf " ucvkuv'leu" *I GP UKRU+."
eqphgtgpeg=ugg"%3; 'lp"C'r r gpf kz-0'

Cv' vj g" eqpenwukqp" qh' [gct" 6." qwt" rcdqtcvqt{" i gpgtcv'gf "cf f kkpccn' i gpg" gzr tguukqp"
o letqcttc{"f cvc"vq"ur gek'kecm'{"vq"eqo r ctg"cpf "eqp'wcu'vj g'dcucn'ngxgn'qh'i gpg"gzr tguukqp"htqo "
y kf "v'r g"O EH9'egm'y kj "vj g"O EH9-7E"cpf "O EH9-4C"r j cug"KK'gpf qet'kp'g'tgukucpv'rkpgu'lp"vj g"
cdugpeg'qh'G₄0Vj ku'lp'hqto cvkqp'ku'ewtgpv'{"dgkpi "wkrk' gf "hqt'vj g"Lqtf cp"rcdqtcvqt{"j qy gxgt"j cu"
cf f kkpccm'{"dggp'ngxgtci gf "lp"[gct"7" f wtkpi "qwt" cpcn' uku'qh'TP CK'uetggp'lpj "f cvc"vq"i gpgtcv'g" c"
hkp'cn'ugt'kgu'qh'vgucdn'g"j {r qvj guguv'vq" f gh'kg"vj g"o ge'j c'pkuo *u'd{"y j lej "G₄lpf wegu'cr qr vuku"lp"
vj g" r j cug" KK' tguukucpv' o qf gn'0Y g" r tgu'p'v' c" f k'uewukqp" qh' ugxgtcn' nc'f'lpj " j {r qvj gugu."
kpeqtr qtcv'kpi 'tgn'gxc'p'v'w'r r qtv'kpi "TP CK'cpf "i gpg"gzr tguukqp" f cvc0'"

Hkpccm'{"y g" r tgu'p'v' hqto cn' TP CK'dcugf "xcn'f'cvkqp" qh' vj g" tgs wkt go gpv' hqt" GUT3" lp"
o gf kv'kpi "G₄/lpf wegf "cr qr vuku"lp"O EH9-7E"egm'wkrk' lpj "vj g"Hgz k'r ncvg'uetggp'lpj "hqto cv'htuv"
f guetkdgf "lp"vj g"[gct"6"tgr qtv'0Vj ku'f cvc"ku'gpv'kgn'{"eqpukucpv'y kj "f cvc"r t'gxk'q'wun'{"i gpgtcv'gf "d{"
vj g"Lqtf cp"rcdqtcvqt{"vq"uj qy "vj cv'lpj kdkkqp"qh'GUT3"hwpevkqp"lpj kdku'G₄/lpf wegf "cr qr vuku"lp"
O EH9-7E0' K6 r qtc'p'v'{"vj ku' f cvc" j ki j rki j w" vj g" tqdw'p'guu" qh' vj ku' cr r tqcej "lp" xcn'f'cvkpi "
cf f kkpccn'ecpf kf cvg'šj ku' gepgtcv'gf "lp"vj g"J V/TP CK'uetggpu0'

Y qtmiCeego r rkuj gf </'Vcunib'e.' [gct'60'

J V/ukTPC'Uetggp'qh'igut qi gp'f gr t'kcvkqp/tgukucpv'O EH9-7E'egm'"

***J gc'vj gt'Ewp'rk'hg.'Rj F.Fcxlf 'C| qtuc.'Rj F'bp'f [qi cpcpf 'Derni wtwpvj cp-+''**

Cu'f guetkdgf "lp"vj g"[gct"6"tgr qtv'."tgr rkecv'g"TP CK'uetggpu'y gtg'r gthqto gf "qp"O EH9-7E"
egm'lp"vj g"r tgu'p'v'g'cpf "cdugpeg"qh'3pO "39/dgc'gut'cf kqn'wkrk' lpj "c"ut'lev'ugt'kgu'qh'vcp'uhgevkqp"
eqpf kkp'pu'r t'gxk'q'wun'{"qr vko k' gf "hqt"vj ku'egm'rkpg"d{"vj g"C| qtuc"rcdqtcvqt{"0C"uecwgr nq'v'qh'vj g"
f cvc'hqt'vj g'Hktuv'f twi i cdng'i gpqo g'uetggp'ku'uj qy p'lp'Hki 0620"

"

"

"

"

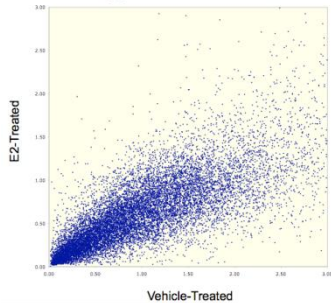
"

"

"

Hli wt g'62"

Viability Ratios of E2 and Vehicle Treated Data:
Druggable Genome Screen 1



Hli wt g'620Pqt o crk gf "t c v k u l h t ' u k T P C ' F I ' u e t g g p ' 3 ' l p ' O E H 9 - 7 E ' e g m 0 3 7 . 2 2 2 " u k T P C " j k u " t g r t g u g p v k p i " 9 2 2 2 " i g p g u 0 R n q w g f " t c v k u " * G 4 / v t g c v g f " x u " X g j k e n g " e q p v t q n " v j c v ' o g g v ' y j g " 6 7 " f g i t g g " c z k u " c t g " v j q u g " r t q v g e v k x g " q h " G 4 / c r q r v q u k u " l p " v j k u " u e t g g p 0 " I g p g u " l p " v j g " n q y g t " t k i j v " s w c f t c p v " c t g " p q v " r t q v g e v k x g " q h " G 4 / o g f k c v g f " c r q r v q u k u 0 " U g x g t c n e c p f k f c v g " j k u " y g t g " @ 2 " r g t e g p v g h g e v k x g " l p " d n q e n k p i " G 4 / k p f w e g f " c r q r v q u k u " c p f " c t g " e w t g p v n { " d g k p i " l p x g u n k i c v g f 0 " " "

Ecpf k f c v g " j k ' k f g p v k l e c v k p " l t q o " t g r n e c v g " T P C k " F t w i i c d n g " i g p q o g " * F I + ' u e t g g p u " * J g c v j g t ' E w p i l l g . R j F ' c p f ' l q i c p c p f ' D c n i w t w p c v j c p + " "

39.4: 6 " f c w " r q l p w " y g t g " q d u g t x g f " l t q o " c " v q c n " q h " h q t v { " h x g " 5 : 6 / y g m " r n v g u " h q m q y k p i " e q o r n g v k p " q h " d q j " F t w i i c d n g " I g p q o g " * F I + ' u e t g g p u 0 V j k u " l p e n w f g f " 8 6 " e q p v t q n " r g t " r n v g " c u " h q m q y u < " "

É54 " T g h t g p e g " y g m u " * e q p v c k p k p i " e g m u . " o g f k c . " p q " u k T P C . " p q " x g j k e n g . " p q " G 4 + "

É: " D r c p n i y g m u " * o g f k c . " p q " e g m u . " p q " u k T P C + "

É: " U e t c o d n g f " E q p v t q n i y g m u " * p g i c v k x g " e q p v t q n i u e t c o d n g f " u k T P C + "

É: " I H R " E q p v t q n i y g m u " * I H R " u k T P C " p g i c v k x g " e q p v t q n i "

É W D D u 3 " y g m u " * r q u k k x g " n g y c n i e q p v t q n i u k T P C + 0 "

F c w " p q t o c r k c v k p " r t q e g f w t g u " y g t g " r g t h q t o g f " c u " f g u e t k d g f " l p " f g v c k i l p " v j g " l g c t " 6 " t g r q t v " c p f " l p " v j g " c r r g p f g f " f q e w o g p v c w j q t g f " d { " F t 0 D c n i w t w p c v j c p 0 V j k u " l p e n w f g f " 8 " n g x g m " q h " f c w " p q t o c r k c v k p < " u c p f c t f " p q t o c r k c v k p . " o g f k c p " p q t o c r k c v k p . " o g f k c p / r q r k u j . " D " u e q t g " c p f " t g i t g u k q p " * 3 ^u " (" 4 ^p " q t f g t + 0 " C h g t " u c p f c t f k c v k p " q h " v j g " f c w . " v j g " u { u v g o " x c t k c d k k v { " y c u " o g c u w t g f " d { " v c n k p i " v j g " f k h g t g p e g " d g v g g p " G 4 " v t g c v g f " c p f " w p v t g c v g f " h q t " v j g " e q p v t q n i " r t q d g u " * T g h t g p e g " c p f " D r c p m i R t q d g u + 0 V j g " l p j g t g p v x c t k c d k k v { " q h " v j g " e q p v t q n i " y c u " c r r n k g f " v q " v j g " t g u v " q h " v j g " r t q d g u " c h g t " c r r t q r t k e v g " n g x g n l u j k v l p " u k i p k h e c p e g " * 3 . " 4 " q t " 5 - 0 C p { " r t q d g u " v j c v y g t g " c d q x g " v j g " u g v " n o k u " y g t g " o c t n g f " c u " e c p f k f c v g " " h i t s " c u " v j g { " c t g " u c v k u k e c m { " u k i p k h e c p v n { " c u u q e k c v g f " * c v " c " w u g t / f g h k p g f " v j t g u j q r f + " y k j " r t q v g e v k p " q h " G 4 / o g f k c v g f " c r q r v q u k u " l p " v j g " O E H 9 - 7 E " e g m u 0 Y g " e j q u g " 3 " c u " q w " n g x g n l u j k u i p k h e c p e g " h q t " u w d u g s w g p v c p c n { u g u 0 "

H q m q y k p i " f c w " p q t o c r k c v k p " v j g " f g h k p g " u k T P C " r t q d g u " v j c v y g t g " p q v e { v q v z k e " c p f " { k r f g f " c " e g m i x l c d k k v { " o g c u w t g " l p " v j g " r t g u g p e g " q h " G 4 " y k j l p " v j g " f g h k p g f " u k i p k h e c p e g " v j t g u j q r f . " y g " f g t k x g f " c " h k p c n i k u v " q h " 4 : ; " e c p f k f c v g " j k u " t g r t g u g p v k p i " 4 9 9 " l p f k x k f w c n i g p g u 0 " Q h " v j q u g " 4 9 9 " i g p g u . " 7 2 i g p g u " o g v " 3 4 B 4 " p q t o c r k c v k p " h c i u " * r c u u g f " c m " 8 " n g x g m " q h " p q t o c r k c v k p " l p " g c e j " T P C " u e t g g p + ; ; " i g p g u " o g v " 3 3 B 4 " p q t o c r k c v k p " h c i u " c p f " 3 4 : " i g p g u " o g v " 3 2 B 4 " h c i u 0 C m i 4 : ; " i g p g u " c t g " t g r q t v g f " l p " V c d n g " 7 " l p e n w f k p i " i g p g u " y k j " y q " u k T P C " j k u " k f g p v k l g f " * v j g t g " c t g " 4 " u k T P C u l i g p g " l p " v j g " F I " u g t k g u + 0 " l p " c f f k k q p " v q " v j g " C F R R Q S " i g p g . " y j k e j " k u " t g r t g u g p v g f " d { " 4 " i g p g " u r g e k h e " u k T P C u " r t l p v g f " o w n k r n g " v k o g u " l p " v j k u " F I " u e t g g p " u g t k g u . " v j g t g " y g t g " 6 " i g p g u " y j g t g " d q j " i g p g / v c t i g v k p i " u k T P C u " t g u w n g f " l p " r t q v g e v k p " q h " G 4 / k p f w e g f " c r q r v q u k u 0 V j g u g " y g t g " O G F 3 6 . " H \ F : . " I T R R 3 " c p f " M H 5 C 0 K " k u " k o r q t c p v " v q " p q v g " v j c v " c m " e c p f k f c v g " j k u " t g s w k t g " h w p e v k p c n i x c r k c v k p p "

y kj "cf f k k p c n i g p g / v c t i g v k p i " u k T P C u " * v { r k e c m { " 6 " u k T P C u " r g t " i g p g " v c t i g v + " j q y g x g t " c p c n { u k u " q h " v j g " e q o d k p g f " h w p e v k p c n " e q p v g z u " q h " v j k u " e c p f k f c v g " r k u " k u " u w h h e k p v " v q " r t q x k f g " p g y " k p u k i j v " k p v q " e q q t f k p c v g n { / f g t g i w r v g f " o g e j c p k u o u " r k n g n { " v q " d g " c u u q e k c v g f " y k j " v j g " G 4 / k p f w e g f " c r q r v q u k u " r j g p q v { r g 0 V j g u g " k p u k i j u " y k n ' d g ' f k u e w u u g f " h w t v j g t " k p " v j k u " t g r q t v 0 ' "

Vcdng'7''

34B4'Pqto cñk cñkq'hei u'	33B4'Pqto cñk cñkq'hei u'	'''	32B4'Pqto cñk cñkq'hei u'	'''	'''
ADIPOQ LIMK1	MARCH2 FSHR	MDM2 RXRA	ACACA EDARADD KIF3A	PLA2G12A	SLC25A43
ADIPOQ LPHN1	ADIPOQ FZD8	MECP2 SFTPB	ADIPOQ EFNA2 KIF3A	PMAIP1	SPARCL1
ADIPOQ MED12	ADIPOQ GADD45B	MED14 SLC2A4	ADCY6 ERCC1 LPO	PPM1E	SQRDL
ADIPOQ MED14	ADIPOQ GALNS	NEDD4L SLC39A6	ADH6 ESRRRA LRAT	PRDX2	SSR1
ADAMDEC1 MUC17	ACTN1 GDF9	NTRK1 SLC5A5	ARHGDIAT EXT2	LRP4	PROP1
AMACR OR6W1P	ANG GH2	ODZ3 SLC6A7	ARNTL FBN1	LTA	PRSS37
ARNT2 OR7D4	APRT GLP2R	OLFM1 SMO	ASB15 FSHB	LYZ	PSMB7
BFAR OR7E5P	C9orf32 GPR161	OPRL1 SNAI2	ASCL1 FZD8	MAML2	PSMC3
BMP7 OR8G2	CA5B GPR173	OR2D2 SPR	ATP6V1A GPR172B	MED16	PSMC5
CRYGD PAPSS1	CASQ2 GPR182	OR5L2 SQRDL	B2M GPR175	MLL2	PTGIR
DEF6 PLEK	CD300LG GPR37L1	OXGR1 TBP	BCL2L10 GPR82	MNAT1	PVRL2
EMR2 PLXNB3	CD79A GPR77	PAPPA TBX5	BCL2L2 GPR89	MOCSS2	RBCK1
ESR1 PPIL2	CETN2 GPX6	PDE6G TMPRSS12	BPIFB1 GRB7	MPP2	RDH8
FAM53B PRPF6	CISH GRM1	PHF23 TMPRSS7	BUB1B GRCA	MRC2	RGS20
FGA PRSS3	COPB2 GTF2H2	PHKG1 TNFAIP8	CAPN5 GRIP1	MTNR1B	RGS3
FGFR1OP2 PTGFR	CORIN HMP19	PIGR TRIM41	CAT GRIP1	MYCBP2	RNF6
FLJ25530 RABGGTB	CPNE4 HNRPUL1	PLCD4 TUBB	CBX4 HCRTR2	NCOA3	ROBO2
GALR2 RIOK3	CRYGA ILK	PLD2 TUBB3	CCL2 HK1	NEB	ROS1
GPR171 SNW1	CTNS ITGA7	PLK3 UBOX5	CDH2 HOXA1	NEURL	RUNX1
GRM6 TFDP2	CYP11B2 ITGB3BP	PML VSIG2	CELSR1 HRK	NPR2	RXFP4
HAS2 TMMEM147	DHFR JAK1	PRPF4B WARS2	CENPB HS3ST1	NR5A1	SAC
HEN2 TREX1	DHRSTC JOSD2	PRSS21 ZNF101	CKM IGBP1	NTF5	SAG
HEG1 TXNDC3	DNASE1L3 KLRG2	PSEN1	CYP2E1 IGF1R	OLIG2	SERPINB13
IL11 VIP	EHD1 LIFR	PTMA	CYP4F8 IL22	OR10AD1	SERPINB6
IL1A VPS39	EPS15L1 LMNA	RAB26	DNAH9 JPH3	OR3A2	SERPINF2
ITGB3 YY1	FBXL18 LOC389772	RAB40C	DSPP KCNH3	PDCD8	SFRP5
LEPROT ZNRF3	FBXO46 MCL1	RARRES2	DUSP16 KIF25	PKM2	SIGLEC5

Vcdng'70'Rtktkk gf "rku"qh'ecpf kf cvg "i gpgu"y j qug"gzr tguakp"ku"rknq "vq"r r { "c"tqng"kp"G4/kpf wegf "cr qr vquku"qh" OEH0<7E"egmu0'Fvc"ku"dcugf "qp"v j g"kpvtugevqp"qh"pqto cñk gf "fvc"ltqo "vy q"ftwi i cdng"i gpqo g"uetggpu0'Ukz" f hgtgpvrgxgn"qh"pqto cñk cñkq"y gtg"r gthqto gf "hqt"gej "uetggp07"i gpgu"uj qy gf "4"qh"4"r quikng"ukTPC"j ku"y kj " v j g'tgo ckpf gt"uj qy kpi "3"qh"4"j ku"cv"v j g'rgxgn"qh"uki pñkcepeg"ugrgev f "3 -0'

O GF 36"ETUR4."FTR372."VTCR392+"ku"cu"uudwvkv"qh"v j g"ETUR"eqhcevqt"tgs vkt gf "hqt" UR3"cevxcvkvq+"eqo r ngz.y j lej "cñkpi "y kj "VHKF."ku"tgs vkt gf "hqt" ghhekvkv"vcpuetkr vqpcn" cevxcvkvq"d { "v j g"UR3"vcpuetkr vqpcn"hevqt0'K"j cu"cnq"dgpp"kf gpvktgf "cu"cu"eqo r qpqp"qh"v j g" o gf kvqt"eqo r ngz"v cv'dtkf i gu'pwenct"j qto qpq"tgegr vqtu"vq"TPC"r qn { o gtcug"KKo cej kpgt { 0Hqt" gzco r ng."Ngg"cpf "eqngci wgu"j cxg"uj qy p"ghhekvkv"eq/cevxcvkvq"qh"Gutqi gp"Tgegr vqt"cnr j c" *GT+"d { "ETUR4"kp" T973"dtgcu"ecpegt"egmu"v tqwi j "cp"NZZNN"r tqvkv"o qvkt"7: +0'K"y qvrf " v j gthqtg"nqi kecm { "hmqy "v j cv"kh"GT"ku"tgs vkt gf "hqt"G4/kpf wegf "cr qr vquku"kp"OEH0<7E."eq/ cevxcvkvq"cuuqekcv f "y kj "GT"cevxcvkv"ctg"rknq "vq"cnq"lo r cev"v j ku"o gej cpkuo 0'kpf ggf."PEQC5" ku"qpq"qh"v j g"i gpg"j ku"fv ggev f "d { "qwt"cpn { uku0Gzr tguakp"qh"ETUR4"ku"pqv"uki pñkcepn { "cnvgtgf " kp"OEH0"xu"OEH0<7E"egmu"i gpg"gzr tguakp"cttc { "fvc"tgr qtv f "kp"l gct"6'tgr qtv."Ewprkktg"rcd-0' "

H\ F: " *tkk | ngf " hco kn { " tgegr vqt " : +0' Htk | ngf " hco kn { " o go dgtu" ctg " tgegr vqtu" hqt " v j g" Y kpi ngu" v { r g" O O VX" kpvi tcvkvq" ukv " *y pv+" hco kn { " qh" uki pcñkpi " r tqvkv" cpf " ctg " v { r kecm { "

eqwr ngf "vq"vj g"dgw/ecvqplp"ecpqpkecn'uki pcrkpi "rcvj y c{0'Vj ku'i gpg"ku'cnuq"rtgf kcvgf "vq"r nc{"cp" ko r qtcvptqng"lp"l /rtqvgkp"eqwr ngf "tgegr vqt'uki pcrkpi "o gej cpkuo u0'kpvgtgukpi n{."vj g"H\ F: "i gpg" ku"38/hqrf "f qy ptgi wrcvgf "cv"vj g"tcpuetr kqpcn'ngxgn'lp"O EH9<7E"egmu'tgrcvkxg"vq"O EH9"y kf " v\rg."cpf "ku'lpf wekdng"d{ "G4"lp"O EH9<7E"dw'pqv'lp"O EH9"egmu'Qwt'f cvc'ui i guu"vj g'r quukdkk\{ " vj cv'G4/lpf wegf "gzr tguukqp"qh'H\ F: "r nc{u'c"o gej cpkuve'tqng"lp"vki i gtłpi "cr qr vquku'qh'O EH9<7E" cu'TPCK'dmencf g"qh'H\ F: "tcpuetr w'ku'r tqvexkxg"qh'G4/lpf wegf "cr qr vquku'0'Vj ku'uco g"ghge'v'ku" pqv'qdugtxgf "lp"O EH9"egmu."vj wu'o c{ "y cttcpv'htvj gt'lp'xguki cvkqp0"

I TR3"*i nweqeqtveqkf "tgegr vqt" kpvgtcevki "rtqvgkp"3+"cnuq"cr r gctu"vq"r nc{"c"tqng"lp" o gfkcvki "G4/lpf wegf "cr qr vquku'qh'O EH9<7E"egmu'0'k'cf f kkp"vq"ku'tqng"lp"o gfkcvki "j qto qpg/ f gr gpf gpv'tcpuetr kqpcn'tgi wrcvqp"d{ "vj g"i nweqeqtveqkf "tgegr vqt."k'j cu'cnuq"dggp"uj qy p"vq" r nc{"c"tqng"lp"j qto qpg"uki pcrkpi "tgi wrcvqp"vj tqwi j "GT"cpf "Gutqi gp/Tgrcvgf "Tgegr vqt"cr j c" *7; +0P q'i gpg"gzr tguukqp'f cvc'ku'cxckcdng'hqt'*I TR3*hqt'O EH9<7E"cu'vj ku'i gpg"ku'pqv'tgr tgugpvgf " qp"vj g"6z66M'r ncvqto 0'

MH5C"*nkpgukp"lco kn{ "o go dgt"5C+"ku"c"uwdwpl'qh'c"j gvgtqtko gtke"o qvqt"rtqvgkp"cpf " r nc{u'cp" ko r qtcvptqng"lp"o letqwdwng"vchlenkpi "qh"rtqvgkp"eqo r ngzgu."pwengle"cekf u'cpf " qti cpnguy kj lp"egmu'0'k'ku'cnuq"vj qwi j v'vq"r nc{"c"tqng"lp"O CRM'uki pcrkpi "cpf "lp"P/ecf j gkp" o gfkcvgf "egm/egm'cf j gukqp0" Gzr tguukqp"qh'*MH5C*"ku"pqv'uki pkhecpv\ "cngtgf "lp"O EH9<7E" tgrcvkxg"vq"O EH9."cpf "*MH5C*"ku'pqv'cp"G4/tgi wrcvgf "i gpg0"Vj g'tqng"vj cv'*MH5C*"o c{ "r nc{"lp"G4/ lpf wegf "cr qr vquku'qh'O EH9<7E"egmu'tgo ckpu'vpergct0'

I gpg'qpvqmi { 'cpcn\ uku'qh'499'Ecpf kf cvg'i gpgu0'

I gpg'qpvqmi { "cpcn\ uku'qh'vj g"vqr "499"ecpf kf cvg"i gpgu'kf gpv'htgf "lp"vj g"TPCKFI "uetggp" uj qy gf "co qpi "uqo g'xgt{ "i gpgtcik gf "I Q"vgtu u+j ki j n{ "uki pkhecpv'cuuqekcvkpu'y kj "rtqeguugu" hqt'*I /rtqvgkp"eqwr ngf "tgegr vqt'uki pcrkpi "*/xcnwg"405"z"32G/9+"cpf "pgi cvkxg'tgi wrcvqp"qh' r tqi tco o gf 'egm'f gevj "*/xcnwg": Q "z"32G/9+0Rgcug"ugg"Vcdng"8"htc"ku'qh'cm'I Q/vgtu u'y kj " GCUG"ueqtg"r/xcnwg">"202230"Vj ku'cpcn\ uku'utqpi n{ "ui i guu"vj gug"hwpevkqpcn'r tqeguugu"ctg" kpxqkxgf "lp"vj g"G4/o gfkcvgf "cr qr vquku'qp"O EH9<7E"egmu'0"*

Vj g"55"i gpgu'tgr tgugpvgf "lp"vj g"ngcf łpi "I Q"vgtu "0I /rtqvgkp"eqwr ngf "tgegr vqt"rtqvgkp" uki pcrkpi "rcvj y c{0'ctg"*I RT383."I RT393."I RT395."I RT3: 4."I RT59N3."I RT99."J OR3;. "UCI . " ADCY6."EGNUT3."CCL2."EKJ . "GOT4."FSHR."HUJ D."H\ F: . "I CNT4."I NR4T."I TO3."I TO8. " JETVT4."NRJ P3."MTNR1B."QT5C4."OPRL1."RNGM"RVI HT. "PTGIR."UO Q."TSHR."TUBB3. " XKR"cpf "XKR40H\ F: "ku'kpenwf gf "lp"vj ku'j ki j n{ "ucv'kuecm{ "uki pkhecpv'qpvqmi { "ui i gukpi "cp" cuuqekcvkqp"qh'vj ku'i gpg"y kj lp"c"dtqcf gt"pgvy qtn'cuuqekcvgf "y kj "f gtgi wrcvgf "I RET"uki pcrkpi 0' Vj g'i gpgu'lp"dqrf ."ctg"cf f kkp'cm{ "cuuqekcvgf "y kj "vj g'I Q"vgtu "0I /rtqvgkp"uki pcrkpi ."eqwr ngf "vq" e{enke'pwergqkf g'ugeqpf "o guugpi gt0'*/xcnwg"9067"z"32G/7."ugg"Vcdng"8+0'*

Vj g"45"i gpgu'qh'kpvgtgu'lp"vj g'I Q"vgtu "0pgi cvkxg'tgi wrcvqp"qh'r tqi tco o gf "egm'f gevj 0" ctg"cu"hqmqy u<"*BCL2L10."BCL2L2."RTQR3."ARHGDI A."XIAP."CTPV4."BFAR."ECV."CCL2. " CBX4."GTEE3."HRK."IGF1R."KVM"IL1A."OPCV3."MCL1."PVTMB."PRDX2."PSEN1."RUOE7. " UO Q*cpf *TNFAIP80* gpgu'lp"dqrf "ctg"cf f kkp'cm{ "cuuqekcvgf "y kj "vj g'I Q"vgtu "0cpv'cr qr vquku0" */xcnwg"304"z"32G/6."ugg"Vcdng"8+0'Z KCR"*DKTE6+"y j kej "ku'cp"łpj kdkqt"qh'cr qr vquku'ku'pqv'cu" j ki j n{ "gzr tguugf "lp"O EH9<7E"egmu'eqo r ctgf "vq"O EH9"*4"hqrf "ngu+."j qy gxgt "Z KCR"cuuqekcvgf "

hcevt "ZKH" y j lej "dlpf u" v" cpf "eqwpgtcew" vj g" kpj kdkqt { " ghgev" qh" ZKR" ku" o cuukgn {
 wr tgi wrvgf "kp"OEHŒE"egm"47/52"hrf +."j qy gxgt"gzr tguukp"qh"ZKH"ku" f qy ptgi wrvgf "d { "G4"
 kp"OEHŒE"uwi i gukpi "vj g"uqlej kqo gvt { "dgvy ggp"vj gug"cr qr vqle"tgi wrvqt { "rtqvgku"o c { "rnc { "
 c"tqrg"kp"vki i gtlpi "G4/kpf wegf "cr qr vuku0J TM" c"DEN4"kpvtcevkpi "rtqvgk"cpf "cevkxcvt"qh"
 cr qr vuku"ku"grgxcvgf "kp"OEHŒE"egm"tgrvkg"vq"OEHŒ"cpf "ku"htvj gt"wr tgi wrvgf "kp"tgur qpug"
 vq"G4"qpni { "kp"OEHŒE0Gzr tguukp"qh"K HBT."y j lej "ku"npqy p"vq"hwpevkp"cu"cp"cpvk/cr qr vqle"
 hcevt "kp" c"pwo dgt"qh"o crki pcpelgu"ku"vgo r qtcni { "o qf wrvgf "d { "G4"kp"OEHŒE"dw'pqv'kp"OEHŒ."
 uwi i gukpi "c" r quukdrg" ng { " tqrg" hqt" vj g" K HBT" uki pcrkpi " ecuecf g" kp" o gf kvkpi " G4/kpf wegf "
 cr qr vuku0DTCT"ku"cnq"cp"kpvtki vkpi "cr qr vqle"tgi wrvqt { "o qrgewg"kp"vj cv'k'rnc { u" c"eqo r rgz "
 dk/hwpevkpcn" tqrg" kp" tgi wrvkpi " kpf wevkp" qh" cr qr vuku" xlc" dqvj " vj g" kpvtkpule" o kqej qpf tkcn"
 o gej cpkuo "i qxgtpgf" d { "Den4"rtqvgku"DTCT"dmem" Dcz/kpf wegf "f gcvj +."cpf "vj g"gzvtpule"
 o gej cpkuo "i qxgtpgf" d { "VP HT" hco kn { "o go dgtu"vj tqwi j "vj gkt" F gcvj "f qo ckpu"DTCT"dmem"
 f gcvj /f qo ckp/kpf wegf "ecur cug"cevkxcvkp+0Vj ku"uwi i guu"vj g"r quukdkk { "vj cv'G4"kp f wegu"cr qr vuku"
 kp"OEHŒE"egm"xlc"dqvj "vj g"kpvtkpule"cpf "gzvtpule"rcvj y c { u0'

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

"

Vcdng'8"

Category	Term	RT	Genes	Count	%	P-Value
GOTERM_BP_ALL	G-protein coupled receptor protein signaling pathway	RT		33	1.3	2.3E-7
GOTERM_BP_ALL	cell surface receptor linked signal transduction	RT		53	2.1	2.3E-7
GOTERM_BP_ALL	negative regulation of programmed cell death	RT		23	0.9	8.9E-7
GOTERM_BP_ALL	multicellular organismal process	RT		102	4.0	9.2E-7
GOTERM_BP_ALL	signal transduction	RT		73	2.8	9.3E-7
GOTERM_BP_ALL	negative regulation of cell death	RT		23	0.9	9.3E-7
GOTERM_BP_ALL	negative regulation of apoptosis	RT		22	0.9	2.8E-6
GOTERM_BP_ALL	second-messenger-mediated signaling	RT		17	0.7	1.0E-5
GOTERM_BP_ALL	regulation of cellular process	RT		134	5.2	2.4E-5
GOTERM_BP_ALL	regulation of programmed cell death	RT		33	1.3	2.7E-5
GOTERM_BP_ALL	regulation of cell death	RT		33	1.3	2.9E-5
GOTERM_BP_ALL	biological regulation	RT		145	5.6	3.0E-5
GOTERM_BP_ALL	negative regulation of cellular process	RT		53	2.1	3.4E-5
GOTERM_BP_ALL	regulation of biological process	RT		138	5.3	4.1E-5
GOTERM_BP_ALL	regulation of apoptosis	RT		32	1.2	5.7E-5
GOTERM_BP_ALL	G-protein signaling, coupled to cyclic nucleotide second messenger	RT		11	0.4	7.4E-5
GOTERM_BP_ALL	negative regulation of biological process	RT		55	2.1	8.5E-5
GOTERM_BP_ALL	anti-apoptosis	RT		14	0.5	1.2E-4
GOTERM_BP_ALL	developmental process	RT		79	3.1	1.5E-4
GOTERM_BP_ALL	multicellular organismal development	RT		73	2.8	1.8E-4
GOTERM_BP_ALL	cyclic-nucleotide-mediated signaling	RT		11	0.4	2.0E-4
GOTERM_BP_ALL	intracellular signaling cascade	RT		39	1.5	4.6E-4
GOTERM_BP_ALL	positive regulation of transcription, DNA-dependent	RT		21	0.8	4.8E-4
GOTERM_BP_ALL	positive regulation of cellular metabolic process	RT		31	1.2	5.2E-4
GOTERM_BP_ALL	positive regulation of RNA metabolic process	RT		21	0.8	5.3E-4
GOTERM_BP_ALL	female gamete generation	RT		7	0.3	5.6E-4
GOTERM_BP_ALL	cell differentiation	RT		46	1.8	6.1E-4
GOTERM_BP_ALL	positive regulation of macromolecule metabolic process	RT		30	1.2	7.2E-4
GOTERM_BP_ALL	anatomical structure development	RT		65	2.5	7.9E-4
GOTERM_BP_ALL	cellular developmental process	RT		47	1.8	7.9E-4
GOTERM_BP_ALL	system development	RT		61	2.4	8.4E-4

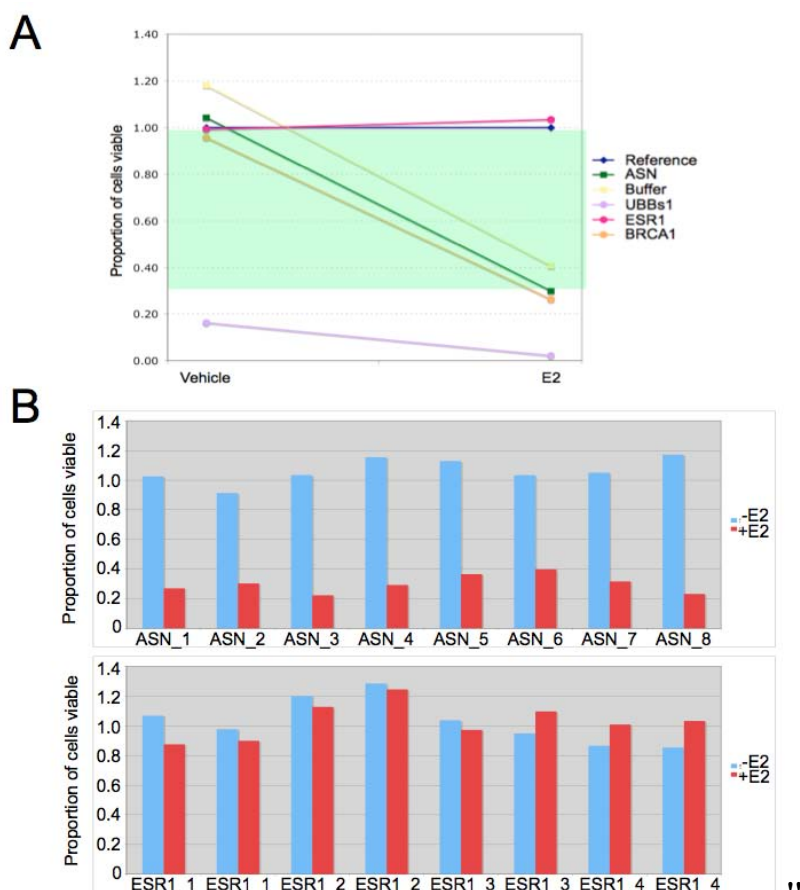
Vcdng'80Vqr "53I Q"vto u"tqo "c"qvcrn'qh'467+"cuuqelcvgf "y kj "vj gecpf kf cvg"rku'qh'499"i gpgu'kf gpv'htgf "lp"vj g'FI "TPCK" uetggp0' " Vj g" I Q" vto u" rku' ku' etqrr gf" cv' r" xcng ">202230' Uqhy ctg" wuf<' F CXKF" GCUG" *j wr <lf cxkf Qdeefeketf qxl+0SEqvpt'"ku'j qy "o cp{"i gpgu'tgr tgu'pvgf "lp"gecj "vto 0R/xcng"ku'c"o qf khtgf "Huj gt'u" Gzcev'R/xcng"qt"GCUG'ueqtg"vj g'uo cmgt"vj g'xcng."vj g'j ki j gt"vj g'gptlej o gpv0'

Xcrkf cvkp'qhtEcpf kf cvg"vj ku'ht qo "vj g'FI "TPCKuetggp0'

C"tcvkqpcrk gf"cti wo gpv'ecp"egtckpn{"dg"o cf g"ht"c"pwo dgt"qh'cf f kkpqpcn'i gpgu'y kj kp"vj gug'I Q"vto u."cpf "kp'ggf "htqo "vj g'ecpf kf cvg"rku'qh'499"i gpgu0'Vj ku'j ki j rki j vu"vj g'pggf "vq" gzr gtlo gpvcn{"xcrkf cvg"vj g'ecpf kf cvg"i gpgu'kf gpv'htgf "lp"vj g"TPCK'uetggp"kp"qtf gt"vq"r tkqtkk g"cp" kp/f gr vj " kpvgttqi cvkp" qh" vj g" o qrgewrct" o gej cpkuo " o quv" rkngrn" vki i gtlpi " G4/kp'wegf " cr qr vaku" kp" vj g" OEH07E" o qf gr0' " C" ewuqo " hgzkr ncvg/eqpcklpi " vj g'ecpf kf cvg"i gpgu' qh" kpvtguy."*eqpcklpi "6"ugr ctcvg"xcrkf cvgf "ukTP Cu'r gt"i gpg"vcti gv'y cu'f gxgnr gf "cpf "r wtej cugf " d{"F t0'C| qtuc's rcdqtcvqt {"cpf "vj g" hgzkr ncvg"TPCK'xcrkf cvkp"uetggp"kp"OEH07E"egmuy cu" r gthqto gf "lp"Ugr vgo dgt"qh'42330"kp"Qevqdg"qh'4233."F t0'C| qtuc"cmgtvgf "o g"vq"vj g"gz vgo go grn"

wphqtwpcvg"pgy u"vj cv"vj gtg"y cu"c"vej pŒcn'o kuj cr "lp"vj g"nd"lp"vj cv"vj g"O EHŒ7E"xcrkf cvkqp" uetggp"j cf "dggp"r gthqto gf "qp"vj g"lpeqttgev"hgzk rŒvg" f gxgnr gf "hqt" c"ugr ctcvg"eqmcdqtcvŒg" uwŒ {0"Vj gtg"y cu."j qy gxgt."3"ecpf kf cvg"i gpg"qp"vj g"lpeqttgev"hgzk rŒvg" wugf "vj cv"qxgtncr r gf " y kj "qwt"ecpf kf cvg"i gpg"nŒv."cpf "vj ku"j cr r gpgf "vq"dg"vj g"i gpg"GUT30"Vj g"xcrkf cvgf "f cv"ku" f gr kevŒf "lp"HŒi wtg"63."cpf "uj qy u"e"erget"tgs vkt go gpv"hqt"GUT3"gzr tguukqp"vq"o gf kvŒ"G4/kpf wegf " cr qr vŒuku"lp"O EHŒ7E0"Vj g"hpŒcn'hgzk rŒvg"xcrkf cvkqp"uetggp"y cu"pqv"eqpf wegf "f vŒ"vq"F t0' C| qtuc's unapvŒkr cvŒf "f gr ctwtg"htqo "VI gp"cv"vj g"gpŒf"qh"42330'

HŒi wtg"63"



HŒi wtg"630Xcrkf cvkqp"qh"GUT3"lp"O EHŒ7E" 1'G4"lp"hgzk rŒvg"htqo cv0C0'XŒcdkŒvŒf "qh"O EHŒ7E"egmŒtgcvgf " y kj "xgj lŒng"qt"G4"hqmy lpi "TP CK'tcpulgevkqp'O Cmi'r qukŒxg"cpf "pgi cvŒxg"eqpvtqni"ctg"uj qy p"cu"y gm"cu"fcv"htq" GUT3"cpf "DTEC30"WDu3"ukTP C"ku"rgv cnŒpq"o cvwt"y j cv"vj g'o gf kv"eqpf kŒqp0Vj g'r tqr qtŒqp"qh'egmŒf gcvj "lp"vj g" CmŒŒct"pgi cvŒxg"eqpvtqni" *CUP + "cpf "dwhgt"eqpvtqntg"uko kŒct"vq"vj cv"qh"DTEC30"EgmŒtgegkŒpi "tcpulgevkqp"qh" GUT3"ukTP C"tgo ckp"xŒcdŒg"lp"vj g'r tgugpeg"qh"G40'DŒ kuqi tco "qh"O EHŒ7E"egmŒxŒcdkŒvŒf "cetquu'o wŒkr Œg"xcrkf cvŒf " ukTP Cu0Vqr "r cpgr"CmŒŒct"pgi cvŒxg"eqpvtqni"uj qy lpi "mŒu"lp"egmŒxŒcdkŒvŒf "y kj "G4"tŒgf "dctun0Dqwo r cpgr"uj qy u"6" ugr ctcvg"ukTP Cu"ci ckpuv"GUT3"*cuuc{ gf "lp"fw rŒcvŒg+uj qy lpi "vj g'r tqvŒvŒxg"ghŒge"qh"GUT3"dmŒenŒf g"*pq"mŒu"lp" egmŒxŒcdkŒvŒf "y kj "G4"tŒgcvo gpv0"

"

Mg{ 'Tgugcte| 'Ceeqo r rku| o gpv|*Hqt 'CmVcum|q|h'v| g'EgG+<

11

Vcum3'*NEEE.'Krcceu+'

- Á kpukwkqpcnrlmpf u'y gtg'ugewt'gf "cv'NEEE'hqt'eqpvpwgf "uwf {"ceetwcn'y j knv'yj g'vkn'y cu' r gto cpgpwnf "enugf "vq"ceetwcn'cv'HEEE"cpf "chhkcvgu."dw'tgo ckpgf "qr gp"cv'NEEE0'
- Á Vj g'r tqveqn'y cu'tcpuklkpgf "vq"NEEE"wpf gt'f'ktgevkqp'qh'Ercwf kpg'Kucceu.'O F 0'
- Á F QF "cr r tqxcn'y cu'qdvckpgf "hqt'c'r tqveqn'co gpf o gpv'y cv'tghngevf "y g'ej cpi g'qh'RKcpf " enquwtg'vq"ceetwcn'cv'yj g"qyj gt'uksgu0'
- Á Uetggpkpi "dgi cp"cv'NEEE"cpf "3'r cvkcpv"j cu'dggp"ceetwgf =uj g'ku'ewttgpwnf "qp"y ggm': "qh' Gutceg"cpf "vqrgetcvpi 'k'y gmf'
- Á Y g"grgevf "vq"o qf kh{"qwt"r tqveqn'vq"8"o i lf cknf."dcugf "qh'h'qh'f cwc"htqo "c"tgegpv' tcpf qo k'gf "Rj cug"KKuwf {"yj cv'yj cf "gzco kpgf "y g"ko r cev'qh'nqy gt/f qug"xgtuwu"j ki j gt/ f qug"gutqi gp"yj gter {"kp"y qo gp"y kj "cf xcpegf "j qto qpg"tgegr vqt'r quklxg'dtgcuv'ecpegt" y j q"j cf "gxkf gpeg'qh'f kugcug'tgukncpeg'vq"ctqo cvcug'kpj kdkqtu."cpf "pqvpi "y g"gutcf knl' f qug'cu'c'dctltgt'vq'r cvkcpv'ceetwcn'cv'yj g'HEEE'uksg0'
- Á Y g"ctg'ewttgpwnf "cy cklpi "F QF "cr r tqxcn'qh'yj g'r tqveqn'co gpf go gpv0'Qpeg"cr r tqxgf." y g'r ncp'vq"gzr cpf "qwt'ceetwcn'vq"qyj gt'uksgu."cu'y gmf'cu'cf xgtvug'yj g'r tqveqn'cpf "kpetgcug" ceetwcn'xlc'yj cv'cxgpwg'cu'y gmf'

"

Vcum'4d/3'¶ Wδ'Lqt f cpHcp+'

- Á Dcugrkpg"ngxgnu"qh'GT."J GT4."cpf"e/Ute"cevkxvqp"y gtg"uwf kfg"lp"e"r cpgn"qh'dtgcuv"ecpegt"egm'rkpgu"y kf /v{r g"O EH/9."V69F."T/97/3."DV696."O F C/O D/453."cpf "UmDt/5+"cpf"gpqetkpg'tgukucpv"O EH/9>E."O EH/9-4C."O EH/9 IH"cpf "V69F -E64-0"
- Á Dcugrkpgu"ngxgnu"y gtg"fhgtgpcvkn'cetquu"y g'r cpgn"cpf "gzr tguugf"ngxgnu"qh'vqcn'e/Ute"cpf r j qur j qt {rvgf "e/Ute0'
- Á Cnj qwi j "y gtg"ku"pq"erget'tgrvqpuij kr "dgw ggp"e/Ute"r j qur j qt {rvkqp"cpf "J T"gzr tguukqp"chgt"pqto cnk gf "d { "vqcn'e/Ute"co qpi "vguvf "egm'rkpgu."y g"qdugt xg"y cv'e/Ute"ku'cevkxcvgf "lp"tgukucpv'egm'rkpgu"eqo r ctgf "y kj "tgur gev xg"r ctgpcv'n'egm'rkpgu"O EH/9>E."O EH/9-4C."cpf "O EH/9 IH"xgtuwu'O EH/9."V69F -E64"xgtuwu"V69F -0'
- Á Vj g'e/Ute'kpj kdkqt."RR4."ghgevkxgn ["dnqengf "GT"pgi cvkxg"dtgcuv"ecpegt"egm'i tqy yj 0"
- Á Vj g"i tqy yj "kpj kdkqt { "ghgew" d { "y j g'e/Ute'kpj kdkqt "qp"GT"r qukkxg"egm'cr r gct "vq"dg"o qtg"eqo r ngz "y cp"qp"GT"pgi cvkxg"egm0"
- Á Kpj kdkkqp"qh'e/Ute"v{tqukpg"nkpcug"r tgf qo kpcpv ["dnqengf "GT"pgi cvkxg"dtgcuv"ecpegt"egm'i tqy yj ."r ctvewrctn ["y j g"vkr ng"Q0GT."RT."cpf "J GT4+"pgi cvkxg"egm0"
- Á Vj g-o clqt k { "qh"GT"r qukkxg"dtgcuv"ecpegt"egm'y gtg"pqv'ugpukxg"vq"RR4"tgi ctf nguu"qh"y kf /v{r g"qt"gpqetkpg'tgukucpv'egm'rkpgu0'
- Á Vj g'e/Ute"chgevgf "y j g'hmpv kqp"qh'GT"lp"GT"r qukkxg"egm0'
- Á RR4"eqwrf "pqv'dnqen"y j g'r tqnhtcvkqp"lpf wegf "d { "G4"lp"O EH/9"cpf "\ T/97/3"egm'dw"r ctvcm ["cdqrkuj gf "G4"unko wrcvqp"lp"V69F"cpf "DV696"egm."lpf kcvkpi "y cv'e/Ute"o k j v"r rc { "c" f krpvctqr "lp"o gf kcvkpi "G4"uki pcnpi "lp"y kf /v{r g"egm0'

- Á Kp"vy q"gpq qetkpg"tgukwcpv"egmu"*OEH/9<7E"cpf "OEH/9<4C+ "vj cv"qxgtgzr tguu"GT."RR4" eqwrf "dmqenlê/Ute"cevxcvkqp"cpf 'cdqkuj gf "cdqww'47' "qh'r tqrlhgtcvkqp"kp "OEH/9<7E"egmu." dwl'y kj qww'cp{ 'kpj kdkkqp"kp "OEH/9<4C"egmu0'
- Á Y g"tgcuqpgf "vj cv"e"eqo dlpvcvkqp"qh"RR4"y kj "G4"y qwrf "gpj cpeg"G4/kpf wegf "cr qr vquku0' J qy gxgt."RR4"f kf "pqv'gpj cpeg"vj g'i tqy vj "kpj kdkqt{ "ghhgewu"qh"G4"qp"vj gug"vy q"egm'lkpgu." dwl'tcvj gt."dmqengf "vj g'i tqy vj "kpj kdkkqp"kp wegf "d{ "G40'
- Á Vj gug"f cxc"ko r rkgf "vj cv"G4/vtki i gtgf "cr qr vquku"o ki j v'dg"wkrl kpi "e/Ute"v{ tqukpg"nkpcug"cu" cp"ko r qtcvpv"uki pcrlpi "r cvj y c{0'Y g"ctg"ewttgpnv "kpxguvi cvkpi "vj g"o gejcpluo u"qh"j qy " vj g'e/Ute"kpj kdkqt"dmqemu"G4/vtki i gtgf "cr qr vquku0'
- Á Cevxcvkqp"ucwuu"qh"J GT4"f gvto kpgf "vj g"kpj kdkqt{ "ghhgewu"qh"vj g'e/Ute"kpj kdkqt0'
- Á J GT4"y cu"j ki j n{ "cevxcvgf "kp"Um/Dt/5"egmu"y j lej "o cf g"kvj { r gtugpuklxg"vq"ncr cvkpkd."c" f wcnv{ tqukpg"nkpcug"kpj kdkqt"qh"J GT4"cpf "GI HT0'
- Á J GT4"j { r gt/cevxcvkqp"tgpf gtgf "dtgcu"ecpegt"egmu"eqo r rgvn{ "tgukwcpv" vq"RR4=" vj g" j ki j gt"J GT"r j qur j qt{ ncvkqp."vj g"ny gt"tgr qpukxg'tcvg"vq"RR40'
- Á Qxgtcm"e/Ute"o gf kvgu"vj g"guugpvkn'tqrg"qh"i tqy vj "r cvj y c{u"kp"GT"pgi cvkxg"dtgcu" ecpegt"egmu"y kj qw"J GT4"qxgt/cevxcvkqp0'
- Á Vj gug"f cxc"r tqxkf gf "cp"ko r qtcvpv"vj gter gwle"tcvkpcrg"lqt"r cvkcpv"ugrgevkv"kp"enkplecn' vlcnu"y kj "e/Ute"kpj kdkqtu"kp"vtr ng'pgi cvkxg"dtgcu"ecpegt0'

Vcuni4d/4'4 W6'Lqtf cpHkp+'

- Á Cpvkuxtqi gpu"6/j { f tqz { nco qzkhgp"cpf "ÆK3: 4.9: 2"dmqem"G4/kpf wegf "cr qr vquku"y j lej " f go qpwtcvf "vj cv'guxtqi gp"wkrl gu"GT"cu"vj g"lpkkn'ukxg"vq"vtki i gt"cr qr vquku0"
- Á Vj g"pqp/tgegr vqt" v{ tqukpg" nkpcug" e/Ute" cevu" cu" c" etkkn'cn' o qrgewrg" kp" tgrn{ kpi " GT" uki pcrlpi ."kpenw kpi "pqpi gpqo le"cpf "i gpqo le"cevqpu0'Ku"cevkv{ "ku"o qf wrcvgf "d{ "G4" vj tqwi j "o wmr ng"o gejcpluo u."ngcf kpi "vq"dtgcu"ecpegt"egm'r tqrlhgtcvkqp."kpxcvkqp."cpf " o gvcucuku0"
- Á e/Ute"y cu"wkrl gf "d{ "G4"vq"kp wegf "cr qr vquku/tgrcvf "r cvj y c{u"kpenw kpi "qz kf cvkxg"utguu" cpf "lphco o cvqt{ "tgr qpugu"gv0"
- Á G4" ecwugf " o kqej qpf tkcn' f { uhwpvkqp" vj tqwi j " f kutw vki " o kqej qpf tkcn' o go dtcpg" kpvgi tkf{ ."hcekrkcvkpi "vj g"tcpuqecvkqp"qh"e{ vqej tqo g"e"htqo "vj g"o kqej qpf tkcn'kv"vj g" e{ vquqn"cpf "kp wekpi "qz kf cvkxg"utguu0"
- Á CR/3"ku"qhvgp"eqpukf gtgf "cu"e"pwerget"f gekukqp/o cngt"vj cv"f gvto kpgu"rhg"qt"f gcvj "egm' hcvgu"kp"tgr qpug"vq"gz tcegmwrt"uko wkl0'Dqj "GT"cpf "CR/3"ctg"pwerget"tcpuetr vkqp" hcevtu0"
- Á Y g"dgrkxg"vj cv"vj g"tqrg"qh"CR/3"kp"cr qr vquku"uj qwrf "dg"eqpukf gtgf "y kj kp"e"eqo r rgz" pgvy qtni"qh" pwerget" hcevtu" vj cv" tgr qpf " uko wncpgqwn{ " vq" c" y kf g" tcpi g"qh" uki pcrl' tcpufr wekv"r cvj y c{u"vki i gtgf "d{ "guxtqi gp0"
- Á CR/3"dgrpi u"vq"dcuke"tgi kqp/ngwepg" | kr r gt" *d\ RR+"r tqvklp" y j lej "ecp"kvgtcev"y kj " pwerget"r tqvklp"P H/nrr c"D"*33+"cpf "qvj gt"d\ RR"tcpuetr vkqp" hcevtu"uwej "cu"ETGD3" *37+0"

- Á K'y kn'dg"cj cngpi g"v"kf gpvk{ "j qy "GT"tgi wrcgu"qt"cuuqekcvgu'y kj "CR/3"vj tqwi j "qvj gt" pwenget"r tqvgkpu"v"tli i gt"cr qr vuku/tgncvgf "i gpgu"lp"npi /vgto "gustqi gp"r gr tkxgf "egmu0"

"

Vcund4d/5'¶ WŒ'Lqtf cpUgpi wr vc+''

- Á Dcucn'gzr tguukqp"qh"eO [E"tcpuetr w"cpf "r tqvgk"ngxgn'ku"5/6"hnrf "j ki j gt"lp"vj g"CK tguukcpv' GT- " dtgcu' ecpegt" egm" o qf gn" OEHŒŒE" egm." cu" eqo r ctgf " v" r ctgpcn' eqwpgtr ctv'O EHŒ'egmu0
- Á J ki j "ngxgn'qh"eO [E"ku"tgur qpukdng"ht"vj g"ustqi gp"lpf gr gpf gpv'i tqy vj "qh"OEHŒŒE" egmu0
- Á Wp/rki cpf gf "ustqi gp"tgegr vqt " "ku"r ctvcm{"tgur qpukdng"ht"vj g"j ki j gt"eO [E"ngxgn'lp" OEHŒŒE"egm"cu"hwkugutcpv'tgcvo gpv."y j kej "f gi tcf gu"vj g"GT "r tqvgk."ecp"r getgcug" vj g"eO [E"ngxgn'cu'y gni'cu"vj g"ustqi gp"lpf gr gpf gpv'i tqy vj "qh"vj gug'egmu0
- Á Tgetwko gpv'qh"j ki j "ngxgn'qh'ugtkg/4/r j qur j qt {rcvgf "TPC"r qn{o gtcug"KK*c"o ctngt"qh" gngpi cvkp"qh'TPC"u{pvj guku"cv"vj g"eO [E"r tqo qvg"lp"OEHŒŒE"egm."cu"eqo r ctgf "v" r ctgpcn'OEHŒ'egm."ku'o quv'kngn{"tgur qpukdng"ht"vj g"j ki j gt"ngxgn'qh"eO [E"tcpuetr wO
- Á J ki j "ngxgn'qh'r j qur j qt {rcvgf /EFM; "ku"hwpgf "lp"OEHŒŒE"egm"y j kej "ku"npqy p"ht"vj g" r j qur j qt {rcvgf "qh'ugtkg/4'tgukf vg"qh'TPC"r qn{o gtcug"KK'
- Á Cevkxcvgf "EFM; "ku"tgur qpukdng"ht"vj g"ustqi gp"lpf gr gpf gpv'i tqy vj "qh"vj g"OEHŒŒE" egm"cu'EFM; "ur gekhe"lpj kdkqt"ecp"dmem'ku"ur qpvcpgquw'i tqy vj O'

Vcund4d/6'¶ WŒ'Lqtf cpUgpi wr vcQdkqt cj +''

- Á Dkur j gpqn'cpf "dkur j gpqn/C."dqj "eqo r qwpf u"cev"cu"cp"ustqi gple"rki cpf "ht"i tqy vj "qh" GT "r qukkxg"dtgcu' ecpegt" egm."y j gtgcu"dkur j gpqn"dw"pqv"dkur j gpqn/C."cew"cu"cp" ustqi gp/cpci qpkv'lp'OEHŒŒE"egm."cu'k'ku'wpcdng"v"lpf weg"cr qr vuku"lp"vj gug'egmu0
- Á Dkur j gpqn*gxgp"cv"j ki j "eqpegpvcvkuu"ecp"qpn{ "r ctvcm{"lpf weg"RU4"i gpg"tcpuetr vkp" cu"eqo r ctgf "v"39/ "gustcf kn'tgcvo gpvOlp"eqpvcuv."dkur j gpqn/C."cv"j ki j gt"eqpegpvcvkuu" ecv'lpf weg"RU4"i gpg"tcpuetr vkp"v"vj g"uco g"gzvgpv'cu"39/ "gustcf kn'tgcvo gpvO
- Á Wulpi "Ej KK"cuuc{."y g"hwpgf "vj cv"cv"j ki j gt"eqpegpvcvkuu"*32⁷O +"dkur j gpqn/C"tgcvo gpv' ecv"tgetwk"GT "cu"y gni'cu"ugtqkf "tgegr vqt"eq/cevkcvgf/5"*UTE5+"r tqvgkpu"cv"vj g"RU4" r tqo qvg"cu"ghhekgpv{ "cu"39/ "gustcf kn'tgcvo gpv'lp"vj g"OEHŒŒE"U: "egmu0Qp"vj g"qvj gt" j cpf."j ki j "eqpegpvcvkuu"qh'dkur j gpqn'hkngf "v"tgetwk"GT "cpf"ugtqkf "UTE5"r tqvgkpu"cv" vj g"RU4"r tqo qvg"cu"ghhekgpv{ "cu"39/ "gustcf kn't
- Á D{ "wulpi "O E4"cpf "LO 8"egm"y g"r gvto kpgf "vj cv'dkur j gpqn'tgs wktgf "vj g"cur ctvcg"co kpg" cekf "cv"vj g"573"r qukkqp"qh'GT "v"lpf weg"vj g"VI H "o TPC"y j gtgcu"dkur j gpqn/C"cpf "39/ "gustcf kn'tkf "pqv'tgs wktg"ku'wi i gukpi "vj cv'o qf g"qh'rki cpf "dkpf lpi "v"vj g"GT "ku'uko krt" ht"dkur j gpqn/C"cpf "39/ "gustcf kn'y j gtgcu'k'ku'f kpkpvn{ "f khtgtpv'ht"dkur j gpqnO
- Á Oqngewct"r qenlpi "uwf lgu"r go qpuxcvf "vj cv'dkur j gpqn'dkpf u"v"vj g"GT "r tqvgk"lp"vj g" uco g"o qf g"cu"6/j {ftqz{ "co qzkhgp"*cpvi qpkv'o qf g+."y j gtgcu'dkpf lpi "qh'dkur j gpqn/C" v"vj g"GT "ku'r tgf kvgf "cu'uko krt"v"39/ "gustcf kn't"r kgy {n'ukndgustqn"ci qpkv'o qf g+O'

O qrgewrct" f qenlpi "uwwf lgu" uwi i guvgf "y gcm'dl p f lpi "ecr cekv "qh" dkur j gpqn/C" cpf "vj cv"kv' ecp"dl p f "vq"GT "lp"vy q" f k v k p e v' o qf gu. "dqj 'r tgf levkxg"qh"ci qpkv'eqphqto cvkp0'

- Á Eqo r tcevkxg"cpn{uku"qh"tgi wrvklp"qh"cr qr vqvk"i gpgu"lp"O EH9<7E"egm" d{ "dkur j gpqn' cpf "dkur j gpqn/C"tgxgcrgf "vj cv'dkur j gpqn'hckrgf "vq"lpf weg"cr qr vqvk"i gpgu"cpf "t gugo drgf " 6/j { f tqz { "vco qz k hgp"vtgcvo gpv'y j gtgcu"dkur j gpqn/C"y cu"cu"cdrg"vq"lpf weg"vj g"cr qr vqvk" i gpgu"cm quv'cu"ghgevkxg"cu"39/ "gutcf kqn'tgcvo gpv0' "

Vcun4d/7'4 W6'Lqtf cp1Qdkqt c j +'

- Á G4/lp f weg f "cr qr vquku" qeewtu"cu" c" f grc { gf " gxgpv" lp"O EH9<7E"egm" lp"eqpvtcu" vq" vj g" i gpgtcm{ "ceegr vgf "pqto 0"
- Á Rcerkczgn" c" e { vq v z k e" e j go qvj gter { . " tcr k f n { "lpf wegu"cr qr vquku"lp"vj g"uco g"egm"nkp g" d { " 46"j tu. "y j krg" G4" dgi kpu"vj ku"r tdegua"chgt"94"j tu" wulpi "c"egm"r tqrkgtcvkp"cuuc { 0"
- Á G4"lpf wegu"GTU" cpf "kphrco o cvqt { "utguu"i gpgu"cu"y gm'cu"cr qr vqvk"i gpgu"vj cv"lpf weg" dqj "vj g"lpvlpule"cpf "gzvlpule"cr qr vquku"r cvj y c { 0"
- Á I kxgp"vj g"cdq xg"tguwmu. "kv"ku"r tqr qugf "vj cv"vj g" f grc { gf "o gej cpluo "qh"cr qr vquku"lpf weg f " d { " G4" kpxqrxgu" cp" kpkkc n { "lpf wevklp" qh" dqj " gpf qr r uo k e" tgvkewwo " utguu" cpf " r tqkphrco o cvqt { "utguu"y kj "gctn { "kpxqrxgo gpv"qh"P QZC" cpf "uwdugs wgpv"cevkvxcp"qh" o kqej qpf tlc n { o gf kvgf "cr qr vqvk"i gpgu"vj cv"rvgt" g z r c p f u" vq" kpxqrxg" qvj gt"cr qr vqvk" i gpgu"lpenmf lpi "vj g" f gcj "tgegr vqt"i gpg" hco k n { 0"
- Á G4/lp f weg f "cr qr vquku" kpxqrxgu" c" pwo dgt"qh"o wmkcevtlc n { gxgpv"vj cv"o c { " g z r n k p" vj g" f grc { gf "cr qr vquku"vj cv"ku"qdugt xgf "lp"vj g"O EH9<7E"egm0"
- Á Rcerkczgn'ugrgevkxgn { "lpf wegu"vj g"VTC KNVP HTUHB2C ID"r cvj y c { "kpkkc m { "y j kej "g z r c p f " vq" kpxqrxg"o qtg" f gcj "tgegr vqtu"y kj "lpj kdkkqp"qh"vj g"egm'e { erg"cvI 3"ej genr kqpvd { "r 430"
- Á P QZC" cpf "F CRMB"cuq"lpf weg f "d { "G4"ctg" g z r tguugf "cv"46"j tu0"Vj g"ko r nkecvgf "tqrg"qh" vj gug" vy q" i gpgu"ku" p q v' q d x k w u n { "cr r ctgpv" vq" vj g" tcr k f " c p f " f g r c { g f " v t g c v o g p v' y k j " r cerkczgn'cpf "G4"t g u r g e v k x g n { 0' "

Vcun5'4 W6'Tlgi gnY gmvglp +'

- Á Kp"O EH9"egm. "Den/4. "c"o clqt"cpvk"cr qr vquku"i gpg. "ku"wr tgi wrvgf "d { "gutcf kqn'tgcvo gpv" y j gtgcu"pq"ej cpi g"qh"Den/4"y cu"uggp"lp"O EH9<7E"egm0'
- Á Qxgtcm"vj g" g z r t g u k q p " c p n { u k u " c p f " r t q v g o k e u " f c v " u j q y " u q o g " l p v g t g u k p i " e q p x g t i g p e g u " g u r g e k m { " k p " c r q r v q v k e " t g i w r v q t { " r c v j y c { u " y j k e j " o c { " d g " h w p e v k q p c m { " t g r x c p v " c u " k p k l c v q t u " q h " g u t c f k q n l p f w e g f " c r q r v q u k u 0'
- Á K'j cu"dgpp"uwi i guvgf "htqo "cp"lp"xktq"uwwf { "vj cv"o kqej qpf tlc"j cxg"dgpp"uki p k h e c p v n { " k p x q r x g f " l p " k p k l c v k p " q h " c r q r v q u k u " l p " v j g " O E H 9 < 7 E " e g m 0' Y g " h q w p f " R J D 3 " c u " q p g " q h " v j g " k p v g t c e v k p i " r c t v p g t u " q h " C I D 3 " e q c e v k x c v q t 0' V q " h w t v j g t " g z r n q t g " v j g u g " h k p f l p i u. " y g " w u g f " O E H 9 < 7 E " w o q t " e g m " l p " c " z g p q i t c h v " o q f g n " v q " g z v g p f " q w t " n p q y n g f i g " q h " g u t q i g p / l p f w e g f " c r q r v q u k u " l p " x k x q 0'

- Á Y g"uj qy "y cv"cv'rgcu"lp"qwt"o qf gn"RJ Du"lpvgtcevkqp"y kj "uki pcn'r tqvklpu"ku" f { pco le." tgi wrcvgf " d { " RVO u." cpf " vko g/f gr gpf gf 0' Vj gug" tguwmu" uwr r qtv' ur gekhlek\ " qh" hqwpf " lpvgtcevkqp0"
- Á Grgxcvgf "rgxgnu"qh"y g"RJ D'r tqvklpu"qhvgp"eqttgrcvg"y kj "o kqej qpf tkcn'f { uhwpvklqp"cpf " eqwrf " dg" wugf " cu" o ctngtu" qh" ko dcrpege" qh" y g" tgr ktcvt { " ej clp0' F wv" vq" y g" eqo r ctwo gpvck\ cvkqp" ghhev." y g" hwpvklqp" qh" y gug" r tqvklpu" y qwrf " dg" gkvj gt" r tq/ i tqy y lwtxlxcn'qt" r tq/cr qr vqve lpgi cvkxg'tgi wrcvtu"qh'egmle { eng0"
- Á O kqej qpf tkcn' r tqj kdklpu" lpvgtcev" y kj "CP Vu" cpf "XF CEu" r tqvklpu" cpf " ucdkkl\ g" y g" Rgto gcdkl\ "Vtcpuklqp"Rqtg"RVR"eqo r rgz0Vj g"RVR'tgi wrcvgu"gpgti { "hmv"cpf " r nc { u'cp" ko r qtvcpvtqrg"lp"lpklcvkpi "cr qr vquku0'
- Á Dqvj " y g" QUV6: " cpf " FCF3" r tqvklpu" ctg" dgrgxgf " vq" dg" uwdvpku" qh" y g" qrk quceej ct { ntcpuhgctug"eqo r rgz0Vj g"rquu"qh"FCF3"hwpevkqp"lpf wegu"cr qr vquku0'Vj g" wy q/j { dtkf "u { ugo u"lpqxqk\pi "FCF3"cu'dck'r qkpw"vqy ctf "y g"O en/3" r tqvklp."qpg"qh"y g" Den/4'hco kn\ "cu"cepf kf cvg'hqt"lpvgtcevkqp0"
- Á Vj g"ugeqpf "i tqwr"qh'r tqvklpu" f gvgvfg "lp"qwt"o cuu"ur gestqo gvt { "cpcn\ uku"qtli kpcvgf "htqo " y g" gpf qr ncuo cvke"tgvewnwo "GT+0'Vj g"GT" r nc { u'cp"ko r qtvcpvtqrg"lp"o clpvc\kpi "y g" e { vquqrke" rgxgn' qh" ecrekwo " cpf " tgi wrcvpi " y g" cr qr vqve" tgr qpug" qh" o kqej qpf tkc0' lpvgtgukpi n\ "qpg"GT"cuuqekcvgf " r tqvklp"Gtnp/4"GT"r k\ kf "tch'r tqvklp+"j cu"c"ugs wvpeg" uko kctk\ "y kj " r tqj kdklpu" cpf " hcm' y kj lp" y g" i tqy kpi " hco kn\ " qh" r tqj kdkl\p" f qo clp/ eqpvck\kpi "RJ D'r tqvklpu."y j lej "lpnwf gu"y g'r tqj kdklpu."y g'uqo cvkpu"cpf "y g'hqvklpu0"
- Á Ctlcl\ k'gv'cn'eqpf wvvgf "i gpg"gzr tguukqp"uwf { "qh"gvstqi gp"lpf wegf "cr qr vquku."eqo r ct\kpi " OEH9"egm' xctkcpw" y cv' y gtg" gvstqi gp/f gr gpf gpv' *Y U: +." qt" tgukwcpv' vq" gvstqi gp" f gr tkcvkqp"4C+."qt"ugpukxg"7E+"vq"G4/uko wrcvgf "cr qr vquku0'Vj g { "hqwpf "uki phkcepv' cuuqekcvkqp"cpf "qxgttgr tgugpvkqp"qh'gpf qr ncuo le"tgvewnwo "utguu"GTU+i gpgu"co qpi " y g'7E/ur gekh"i gpgu0Vj ku'hpmlpf kcvgf "y cv'G4\kpj kdkgf " r tqvklp"hnf kpi "cpf "tcpuwcvkqp0'
- Á Vj g" o clqt" tgvtkvklqp" hqt" uwf { " cpf " gxcnvcvklqp" qh" ppxgn' r j qur j qt { rvcvklqp" ukgu" ku" y g" cxckrdkl\ "qh"ko o wpqej go kcn'tgci gpw."uwej "cu'r j qur j qur gekh"cpvkdqf lgu'hqt"Y guvgtp" dmqv\kpi 0' Vq" qxgteqo g" y ku" rko kcvkqp." y g" ko r ngo gpvgf "cp" cuuc { "qp" y g" dcug" qh" y g" NE OTO/O U'r ncvqto "v"gpcdrg"y g'o gcuwtgo gpv'o gyj qf 0"

Vcunl6*VI gp'6'C\ qt uc lDcni wt wpcvj cpEwpklHg+''

- Á Y g"j cxg"eqo r ngvgf "tgr ncevgn'lpvcug"TP CK'uetggpu."cpf "tgr ncevgn'f twi i cdng'i gpqo g"FI + TPCK'uetggpu"lp"OEH97E"egm'lp"y g'r tgugpeg"cpf "cdugpeg"qh'gvstqi gp0'
- Á Y g"j cxg'kf gpvkl\gf "499"ecpf kf cvg"i gpgu"y cv'ctg'r tqvgevkxg"ci clpuv'G4/o gf kcvgf "cr qr vquku" lp"OEH97E"egm'htqo "y g'FI "TPCK'uetggp0'
- Á I gpg"gzr tguukqp"o letqcttc { "uwf lgu"j cxg"dgpg"eqpf wvvgf "vq"kf gpvkl\ "y g"dcucl'rgxgn'qh" gzr tguukqp"qh'cm'i gpgu"lp"y g"OEH97E"cpf "OEH94C"egm'hpgu"eqo r ctgf "vq"y kf "v'r g" OEH9"egm'lp"y g"cdugpeg"qh'G40Vj ku'y qtnly cu'cee qo r rkuj gf "vq"go r qy gt"lpvgi tcvkqp"qh" o wnkrg" cpcn\ vcecl' cr r tqcej gu" d { " y g" Lqtf cp" rcdqtcvt { ." cpf " vq" cuukv' y kj " hwtj gt"

r tkqt k k c v k p " q h " e c p f k f c v g " i g p g u " k f g p w h g f " d { " v j g " T P C K " u e t g g p k p i " h q t " u w d u g s w g p v " x c r k f c v k p 0 "

- Á C m ' i g p g " g z r t g u k p " o k e t q c t t c { " u w f l g u " r g t h q t o g f " d { " v j g " L q t f c p " N e d q t c v q t { " P q t v j y g u v g t p " W p k x g t u k { " O k e t q c t t c { " E q t g " H e k k v { + " c p f " v j g " E w p r k h g " N e d q t c v q t { " y g t g " x c p u h g t t g f " v q " c " 3 V D " g z v g t p c n ' j c t f / f t k x g " c p f " t g w t p g f " v q " v j g " L q t f c p " N e d q t c v q t { " r t k q t " v q " v j g " e q o r r g v k p " q h ' v j g " F q F " l g c t " 7 " d w f i g v r g t k q f 0 "

T g r q t v c d n g ' O w e q o g u < ' "

R w d i n e c v k p u ' "

- 30 Á [c p i " E \ . " [c p k i g t " U K " L q t f c p " X E . " M r g k p " F L " D k w p g t " I F 0 ' O q u v " R r c u k e " R t q f w e u " T g r c u g " G u t q i g p k e " E j g o k e c n < " C " R q v g p k e n " J g c n j " R t q d r g o " V j c v " E c p " D g " U q r x g f 0 ' G p x k t q p o g p v c n ' J g c n j " R g t u r g e v k x g u " 4233 < 33 ; * 9 < : ; / ; ; 80 '
- 40 Á L q t f c p " X E . " Q d k q t c j " K " H c p " R . " M k o " J T . " D t c w e j " J 0 ' G x q n w k p " q h " N q p i / V g t o " C f l w x c p v " C p v k / j q t o q p g " V j g t c r { < " E q p u g s w g p e g u " c p f " Q r r q t w p k k g u < " V j g " U 0 ' I c m g p " R t k g " N g e w t g 0 ' D t g c u v " 4233 < 42 * U w r r n 5 + U 3 / U 330 '
- 50 Á L q t f c p " X E 0 ' F g e c f g u " q h " F k u e q x g t { < " V j g " U g r g e v k x g " G u t q i g p " T g e g r v q t " O q f w r v q t " * U G T O + " U q t { < " V j g " U 0 ' I c m g p " R t k g 0 ' T g l g t g p e g u " g p " I { p g e q r q i k g " Q d i a g t k s w g " 4233 < 36 < 5 : 7 / ; 40 '
- 60 Á J w \ \ . " M e i c p " D N . " C t k k | K G . " T q u g p y c n F U . \ " j c p i " N . " N k L X . " J w c p i " J . " Y w E . " L q t f c p " X E . " T k g i g n C V . " Y g m u v g k p " C 0 R t q v g q o k e " c p c n { u k u " q h ' r c v j y c { u " k p x q r x g f " k p " g u t q i g p / k p f w e g f " i t q y v j " c p f " c r q r v q u k u " q h ' d t g c u v " e c p e g t " e g m 0 ' R N q U Q P G " 4233 < " G r w d " 8 * 8 + g 426320 '
- 70 Á N g y k u / Y c o d k ' L U . " M k o " J . " E w t r c p " T . " I t k i i " T . " U c t n g t " O C . " L q t f c p " X E 0 ' V j g " p g y " u g r g e v k x g " g u t q i g p " t g e g r v q t " o q f w r v q t . " d c | g f q z k h g p g . " k p j k d k u " j q t o q p g / k p f g r g p f g p v " d t g c u v " e c p e g t " e g m i t q y v j " c p f " f q y p t g i w r v g u " g u t q i g p " t g e g r v q t " " c p f " e { e n k p " F 30 ' O q r g e w r t " R j c t o c e q r q i { " 4233 < : 2 * 6 + 832 / 8420 '
- 80 Á I w r c . " U 0 ' R t q h k g " q h " X 0 ' E t c k i " L q t f c p 0 ' R t q h k g " q h " c " t g e g p v n { " g r g e v g f " o g o d g t " q h ' v j g " P c v k p c n ' C e c f g o { " q h " U e k p e g u " v q " c e e q o r c p { " v j g " o g o d g r ' u " k p c w i w t c n ' C t v k e r g 0 ' R t q e g g f k p i u " q h ' v j g " P c v k p c n ' C e c f g o { " q h " U e k p e g u " W U C 04233 < 32 : * 69 + 3 : : 98 / 3 : : 9 : 0 '
- 90 Á C t k k | K ' G C . " E w p r k h g " J G . " N g y k u / Y c o d k ' L U . " U n k n g t " O L " Y k r k u " C N . " T c o q u " R . " V c r k c " E . " M k o " J T . " [g t t w o " U . " U j c t o c " E I P . " P k e q r u " G . " D e m i w t w p c v j c p [. " T q u u " G C . " L q t f c p " X E 0 ' G u t q i g p / k p f w e g u " C r q r v q u k u " k p " G u t q i g p " F g r t k x c v k p / t g u k n c p v " D t g c u v " E c p e g t " x k c " U t g u u " T g u r q p u g u " c u " K g p w h g f " d { " I n q d c n ' I g p g " G z r t g u k p 0 ' R t q e g g f k p i u " q h ' v j g " P c v k p c n ' C e c f g o { " q h " U e k p e g u " W U C 04233 < 32 : * 69 + 3 : : 9 ; / 3 : : 80 '
- : 0 Á R q k t q v " O 0 ' H q w " f g e c f g u " q h ' f k u e q x g t { " k p " d t g c u v " e c p e g t " t g u g c t e j " c p f " t g c v o g p v ' o " c p " k p v g t x k g y " y k j " X 0 ' E t c k i " L q t f c p 0 ' k p v g t p c v k p c n ' L q w t p c n ' q h ' F g x g r u r o g p v c n ' D k q r q i { " 4233 < 77 < 925 / 9340 '
- ; 0 Á Q d k q t c j " K G . " L q t f c p " X E 0 ' R t q i t g u u " k p " g p f q e t k p g " c r r t q c e j g u " v q " v j g " t g c v o g p v " c p f " r t g x g p v k p " q h " d t g c u v " e c p e g t 0 O c w t k c u " 4233 < 92 < 537 / 5430 '
- 320 Á c z k o q x " R [. " L q t f c p " X E 0 ' * 4234 + " G u t q i g p / k p f w e g f " C r q r v q u k u " k p " D t g c u v " E c p e g t " E g m u < " V t c p u r v k p " v q " E n k p l e c n ' T g r g x c p e g 0 ' k p < " V c t i g v k p i " P g y " R c v j y c { u " c p f " E g m i " F g c v j " k p " D t g c u v " E c p e g t " * T g d g e e c " C h v * G f + 0 ' k p V g e j . " T k l g n e . " E t q c v k c . " r r " 5 / 440 '

- 330Áy gpgg{"GG."O eF cplgn"TG."O czko qx"R[."Hcp"R" cpf "Lqtf cp" XE" *4234+" O qf gnu" cpf " O gej cpluo u"qh" Ces vktgf "Cpvj qto qpg" Tgukvcpv"kp" Dtgcuv" Ecpegt< "Uki phkecpv" Enkplecn" Rtqi tguu" F gur kg" Nko kcvkpu0" J qto qpg" O qngewct "Dkqmi { "cpf "Enkplecn" kpxguki cvkqp"; 365/ 850'
- 340Á cuu"O NU."O cpuqp"LG."Equo cp" H" I tqf uvkp" H" Lqtf cp" XE." Mctcu" TJ ." Mcwvki " CO ." O cnk" RO ." Uej o kf v" RL" Uj ktgp" LN." Uwgpngn" EC." Wkcp" Y J 0*4234+" Rqukkqp" Ucvgo gpv< "Vj g" 4234" J qto qpg" Vj gter { " Rqukkqp" Ucvgo gpv" qh" Vj g" P qtvj " Co gtkecp" O gpqr cwug" Uqekgv{ 0' O gpqr cwug< "Vj g" Lqwtpcn" qh" Vj g" P qtvj " Co gtkecp" O gpqr cwug" Uqekgv{ 3; 479/930'
- 350Á g" J J ." O g{ gt" EC." Ej gp" O Y ." Lqtf cp" XE." Dtqy p" O ." Nkw" Z U" F khtgtpvkn" F P cug" K j { r gtugpukxkv{ " tgxgcu" hcevq/ f gr gpv gpv" ej tqo cve" f { pco leu0' I gpqo g" Tgugctej " 4234" *Grwd" c'j gcf " qh'rtkp v0'
- 360Á lqtf cp." XE0*4234+" Gvtqi gp" Cevkqp." UGT O u" cpf " Y omen' u" J gcmj 0' Y qtrf " Uekgpv hle0' P gy " Lgtug{ . " Nqpf qp." Uki cr qtg" *kp'rtgrctcvkqp+0'
- 370Á lqtf cp." XE" cpf " O czko qx" R[" *4234+" Vco qz khp/ Rkqpggtkpi " O gf lekpg" kp" Dtgcuv" Ecpegt0' O kguvqpgu" kp" F twi " Vj gter { 0' Ugtkgu" Gf u< " Rctpj co " O L" cpf " Dtwkpxgnu" L0' Ur tkpi gt " Dcuqn" CI ." Dcuqn" Uy k{ gtrcpf " *kp'rtgrctcvkqp+0'
- 380Áy gpgg{ " G" cpf " Lqtf cp" XE0' *4234+" Gvtqi gp" Tgegr vqt " *GT +0' k< " Gpe { enmr gf kc" qh" Ecpegt" Vj gter gwke" Vcti guu0' Ur tkpi gt " Uekpeg" - " Dwukpgu" O gf kc. " NNE." P gy " I qtni" *kp'rtguu+0'
- 390Á O eF cplgn" T." Lqtf cp" XE0' *4234+" Gpf qetkpg" Rtgxgpvkv" qh" Dtgcuv" Ecpegt0' k< " Gctn{ " Dtgcuv" Ecpegt< " Htqo " Uetggkpi " vq" O wnkf kuek nkpt { " O cpci go gpv0' kphqto c" J gcmj ectg. " Nqpf qp." Gpi rcpf " *kp'rtguu+0'
- 3: 0Á kcp" R." O eF cplgn" TG." Mko " J T." Emi gw" F." J cffcf " D." Lqtf cp" XE0' *4234+" O qf wrcvki " Vj gter gwke" Ghgeu" qh" vj g" e/ Ute" kbj kdkqt" xlc" Gvtqi gp" Tgegr vqt " cpf " J GT4" kp" Dtgcuv" Ecpegt" Egni Nkpgu0' Gvtqr gcp" Lqwtpcn" qh" Ecpegt " *kp'rtguu+0'

Cduntcev'

- 30Á Cduntcev" % 77" y cu" rwdrkuj gf " kp" vj g" 4234" Rtqeggf kpi u" qh' vj g" 325^{tf} " Cppwcn" O ggkpi " qh' vj g" Co gtkecp" Cuqekcvkqp" lqt " Ecpegt " Tgugctej . " Ej keci q. " KN." O ctej " 53/ Crtkl6. " 42340' Tqrg" qh' eO [E" cu' c" et kkecnf gvgto kpcpv' qh' gvtqi gp/ kpf gr gpv gpv' i tqy vj " qh' gvtqi gp" f gr t kcvkqp/ t gukvcpv' dt gcuv' ecpegt ' egm0' Uwtqlggv" Ugpi wr v. " O lej cgn" Dktpgu. " X0Etcki " Lqtf cp0'
- 40Á Cduntcev" % 78" y cu" rwdrkuj gf " kp" vj g" 4234" Rtqeggf kpi u" qh' vj g" 325^{tf} " Cppwcn" O ggkpi " qh' vj g" Co gtkecp" Cuqekcvkqp" lqt " Ecpegt " Tgugctej . " Ej keci q. " KN." O ctej " 53/ Crtkl6. " 42340' Vj g' hkg/ Z/ tgegr vqt / ' k' lpxqngf ' kp' vj g' kpf wcvkqp' d{ ' Vco qz khp' qh' dt gcuv' ecpegt ' egm' f khtgtpvkvkqp' bpf ' f gcvj 0' I tgi qt { " Ugi cr. " Rj kkr r g" f g" O gf kpc. " O lej cgn" Rckmruug. " X0Etcki " Lqtf cp. " Ucpf tkpg" Uxkgyv/ Rqktqv. " O cte" Rqktqv0'
- 50Á Cduntcev" % 4; 43" y cu" rwdrkuj gf " kp" vj g" 4234" Rtqeggf kpi u" qh' vj g" 325^{tf} " Cppwcn" O ggkpi " qh' vj g" Co gtkecp" Cuqekcvkqp" lqt " Ecpegt " Tgugctej . " Ej keci q. " KN." O ctej " 53/ Crtkl6. " 42340' Cffkktqpcmf . " vj k' cduntcev' y cu' ugrgevgf " cu' cp" qt cn' rt gupvkvkqp' kp" c" O kpkul o rqukw " uguukqp0'

Etlkēcn' o gf kēvkqp" qh' G4/lpf wegf " cr qr vquku' vj tqwi j " e/Ut e" lp" mpi /vgt o " gumt qi gp" f gr tkxgf 'dt gcu'ēcpegt 'ēgm0'

Rkpi 'Hcp.'Qdk'N0I tkhkj . 'Rcxpc' Cpwt. 'J grgp' T0Mko . 'Lqg' Y 0I tc{ . 'X0Etcki 'Lqtf cp0'
 60Á Cduat cev"%δ; 46"y cu"rwdrkij gf "lp"vj g"4234"Rt qeggf kpi u"qh'vj g"325^{tf} "Cpwwcn'O ggkpi "qh'vj g" Co gtkecp" Cuuqekēvkqp" hqt" Ecpegt" Tgugctej . " Ej kēci q. " KN. " O ctej " 53/Crtkl' 6. " 42340' Cff kēkpcnē . "vj ku'cdiat cev'y cu"ugrēvgf "cu"pgy uy qt vj { "hqt "vj g"CCET"Cpwwcn'O ggkpi . "cpf " rctvqmiēlp"vj g'o gf kē"qwt gcej 'rt qeguu. 'lpemf kpi 'rt guu't grgcugēqplgt gpeg"cevkkēku0'
Cngtēvkqp"qh'vj g'uj cr g'qh'vj g'ni cpf "gumt qi gp"t gegrvqt "eqo r ngz"eqpvt qni'gumt qi gp/ lpf wegf " cr qr vquku'lp' dt gcu'ēcpegt 0'

Kg{ lpy c'Qdkqtcj . 'Uwtqlggv'Ugpi wr c. 'Tco qpc'Ewtr cp. 'X0Etcki 'Lqtf cp0'
 70Á Cduat cev"%77; 9"y cu"rwdrkij gf "lp"vj g"4234"Rt qeggf kpi u"qh'vj g"325^{tf} "Cpwwcn'O ggkpi "qh'vj g" Co gtkecp" Cuuqekēvkqp" hqt' Ecpegt' Tgugctej . 'Ej kēci q. 'KN. 'O ctej "53/Crtkl'6. '42340'
O qf wēvkpi "vj gt cr gwle'ghgevu'qh'e/Ut e'lpj kēkqt 'xkē "gumt qi gp"t gegrvqt "cpf "J GT4'lp" dt gcu'ēcpegt 'ēgmilēpau0'

Rj kkr r 'l 00 czko qx. 'Twaugni'G00 eF cplēn'X0Etcki 'Lqtf cp. 'J kntw' Dtcvej 0'
 80Á Cduat cev"%78; 4"y cu"rwdrkij gf "lp"vj g"4234"Rt qeggf kpi u"qh'vj g"325^{tf} "Cpwwcn'O ggkpi "qh'vj g" Co gtkecp" Cuuqekēvkqp" hqt' Ecpegt' Tgugctej . 'Ej kēci q. 'KN. 'O ctej "53/Crtkl'6. '42340'
O qf gēkpi "vj g'r j cto ceqni kēcn'lo r qt vpep" qh'gpf qzēkp" hqt "vj g"vt gcvo gpv'qh'GT/ r qukkēg' dt gcu'ēcpegt 'lp' t t go gpqr cwēnē cēlēpau0'

Rkpi 'Hcp. 'Twaugni'G00 eF cplēn'J grgp' T0Mko . 'X0Etcki 'Lqtf cp0'

Rt gupvcēkpu'

4233<

30Á Lqtf cp" XE" δGxqnēvkqp" qh" mpi /vgt o " cf lwxcpv" cpwēj qto qpg" vj gter {< eqpugs wēpegu" cpf " qrr qtwpkēku0' Vj g" 4233" U0' I cnēp" Rtē g" lp" Enēplecn' Dtgcuv' Ecpegt" Tgugctej " Ngēwtg0' Tgugctej " Wf cvē" Ugo kēct" Ugtēgu. " Nqo dētf k' Ego r tēj gpukēg" Ecpegt" Egpvt. " I gqti gvqy p" Wpēkēgtukē { 'O gf kēcn' Egpvt. " Y cuj kpi vqp. 'F E. 'Lwn{ '35. '42330'

40Á Y gēkgt "N. 'Lqtf cp" XE0δ0 kēkqp" cpf 'O cpf cvē" hqt "vj g" Eqpē wēvu'qh' Ecpegt "lp"vj g" Wpēkēg' "Ucēgu" qh' Co gtkec0' Tgugctej " Wf cvē" Ugo kēct" Ugtēgu. " Nqo dētf k' Ego r tēj gpukēg" Ecpegt" Egpvt. " I gqti gvqy p" Wpēkēgtukē { 'O gf kēcn' Egpvt. " Y cuj kpi vqp. 'F E. " Ugr vgo dgt "36. '42330'

50Á Lqtf cp" XE0δ0 kē r tqxkpi "vj g" Tgur qpug' Tcēgu" vq" Gpf qetēpēg' Vj gter { 0"35^y "Cpwwcn'N{ pp" Uci g" Dtgcuv' Ecpegt" U{ o r qukwō . 'Ej kēci q. 'KN. " Ugr vgo dgt "37/3: . '42330'

60Á Lqtf cp" XE0' δVj g" eqpugs wēpegu" qh" qgumt qi gp/ f gr tkēvkqp" lp" dtgcuv' ecpegt" vt gcvo gpv< qgumt qi gp/ lpf wegf " cr qr vquku0' Mētrēkpūē " Kpukēwg. " Uqēnj qm . " Uy gf gp. " Ugr vgo dgt " 48. " 42330'

70Á Lqtf cp" XE0δWpcpēkē cvēg' "Qrr qtwpkēku" hqt "vj g" Vtgcvo gpv' cēp' "Rt gēgpēkqp" qh' Dtgcuv' Ecpegt" y kēj " Rj { ukēni kē " Gumtēf kēn' Vj gter { 0' P qtvj " Egcuv' Dtgcuv' Ecpegt" Eqpēgtēgpeg" 4233. " Ucpf wumē . 'QJ . 'Qēvqdgē "9/: . '42330'

- 80ÁLqtf cp"XE0'õF gecf gu"qh" F kœqxtg { "lp"vj g"Vtgcvo gpv"cpf "Rtgxgpvkqp"qh"Dtgcuv"Ecpegt"d { "vj g" O qf wœvkqp"qh"Gutqi gp"Cevkqp0"P qtvj "Eqcu"Dtgcuv"Ecpegt"Eqphgtgpeg"4233."Ucpf wumf ."
QJ ."Qevqdtg"9/ : ."42330'
- 90ÁLqtf cp" XE0' õkpxgukl cvkqp" kpq" vj g" o qf wœvkqp" qh" gutqi gp/kpf wegf " cr qr vuku0' Erkplecn'
Tgrxcpegö"Ucpf "Wr "4"Ecpegt"*UW4E+."4^{pf}"CCET"Dtgcuv"Ecpegt"F tgco "Vgco "*"DEF V+"
Wf fcvg'O ggkpi . 'F cmu."VZ."Qevqdtg"43/44."42330'
- : 0ÁLqtf cp"XE0'õJ qto qpg"tgegr vqt"r qukkxg"f kugcug"uudi tqwr "w f cvg"*tgegpv'r tqi tguu0'Ucpf "
Wr "4"Ecpegt"*UW4E+."4^{pf}"CCET"Dtgcuv"Ecpegt"F tgco "Vgco "*"DEF V+"Wf fcvg'O ggkpi . "
F cmu."VZ."Qevqdtg"43/44."42330'
- ; 0ÁLqtf cp"XE0'õVj gp"cpf "P qy <"Hqwt" F gecf gu"qh" F kœqxtg { "lp"Dtgcuv"Ecpegt"Vtgcvo gpv"cpf "
Rtgxgpvkqp0"Uctcj "Ugy ctv' Ngewtg" Ugtkgu."I gqti gvqy p" Wpkxgtukv{ "Uej qqn' qh" O gf lekpg."
I gqti gvqy p" Wpkxgtukv{ "O gf lecn'Egpvtg."Y cuj kpi vqp."F E."P qxgo dgt"39."42330'

4234<

- 1.ÁLqtf cp" XE" õO qf wœvkpi " Gutqi gp" Tgegr vqt" Cevkqp" kp" Dtgcuv" Ecpegt<" Vj g" I qqf o cp" (" I kœ cp" Cy ctf " hqt" Tgegr vqt" Rj cto ceqmj { 0' Vj g" Co gtkecp" Uqelgv{ " qh" Gzr gtlo gpvcn' Rj cto ceqmj { " cpf " Vj gter gwleu" *CURGV+0' Tgugctej " Wf fcvg" Ugo kpct" Ugtkgu." Nqo dctf k' Ego r tgi gpukxg" Ecpegt" Egpvtg." I gqti gvqy p" Wpkxgtukv{ " O gf lecn' Egpvtg." Y cuj kpi vqp." F E." Lcpwct { "3: ."42340'
- 2.ÁLqtf cp" XE" õVj g" UGTO "Uqt { "hqt" Dtgcuv" Ecpegt" (" Dg{ qpf "*"Uqo gyj kpi "Qw"qh" P qvj kpi +0' Nkpm' Ngewtg. "Vj g" Kpukwng"qh" Ecpegt" Tgugctej . "Tq{ cn' O ctuf gp" J qur kcn" Nqpf qp." Gpi rcpf . " O ctej "37."42340'

Cy ctf u'œpf 'J apqtct { 'O go dgtuj kr u'

- 30ÁCrthl'4: ."42340' X0' Etcki "Lqtf cp" cy ctf gf "vj g"4234"Nqwu"U0' I qqf o cp" (" Crhtgf "I kœ cp" Cy ctf " kp" Tgegr vqt" Rj cto ceqmj { " htqo " vj g" Co gtkecp" Uqelgv{ " hqt" Rj cto ceqmj { " cpf " Gzr gtlo gpvcn' Vj gter gwleu" *CURGV+" kp" Ucp" F kgi q." EC0' Vj ku" cy ctf " tgeqi pl gu" cpf " uko wœvgu"qwucpf kpi "tgegr vqt" "kp"r j cto ceqmj { "qh'dkqmj lecn'tgegr vqtu."y j lej 'y qwf "rgcf "vq" c"dgwgt"wpf gtucpf kpi "qh'vj g'o gejc pkuo u'qh'dkqmj lecn'r tqeguugu"cpf "r qvgpvcnm{ "r tqxkf g"vj g" dcuku'hqt"vj g"f kœqxtg { "qh'f twi u'wughwilkp"vj g"vtgcvo gpv'qh'f kugcugu0'

Crrqlpwo gpvu'

- 30ÁO lej cgrE0Dlctpgu'DU' Lwnf '8."4233."Tgugctej 'Cuukncpv'K
- 40ÁHcf gng'Ci dqng.'DU' Cwi wuv'53."4233."Tgugctej 'Cuukncpv'K
- 50ÁRwrcplcnKDj cwc.'DU' Ugr vgo dgt"36."4233."Tgugctej 'Cuukncpv'K
- 60ÁCo { 'Dqvgm'
- Cr tkl'52."4234."Gzgewkxg'Cuukncpv'vq'F t0X0'Etcki "Lqtf cp"

EQPENWUKQPU'

K'ku"ko r qtcvpv"vq"utguu"cv"vj g"qwugv"qh"vj ku"ugevqp."vj cv"vj gtg"ku"i tqy kpi "o qo gpwo " y kj kp"vj g"enplecn'eqo o wplv{ "vj cv'qwt"y qtm'ku"cp"ko r qtcvpv'pgy "f ko gpukqp"kp"y qo gn'u"j gcmj 0' Vj ku'ku'kmwutcvgf "d{ "vj tgg."y gm'f ghkpgf "hcew"'

3+Á Qwt"hqewu"qp"vj g"cr r necdkkv{ "qh"qwt"ncdqtcvqt { "tguwu"vj tqwi j "vj g"wug"qh"mly "f qug" gwtqi gp" hqt" vj g" vgcvo gpv" qh" o gvcucvle" dtgcu" ecepgt" hqmly kpi " cpvj qto qpg" f twi " tgukucpeg" ku"pqy "c"i gpgtcn' qv le" qh" f kuwukqp0' Qwt" y qtm'j cu" dggp" r kxqcn' hqt" vj g" r wdncvqp"qh"qvj gtu"vj g"eqpegr v'y g"r tqr qugf "htqo "vj g"ncdqtcvqt { "f cv" gpj cpegu"vj g" vgcvo gpv'qh'y qo gp'y kj "dtgcu'ecpegt" *5.'82+0'

4+Á Qwt"eqpegr w"htqo "vj g"dcuku"qh"o clqt"enplecn'vlecn'kp"Gwtqr g"cpf "ctqwpf "vj g"y qtrf ." f guetkdgf "cu"vj g"Uwf { "qh'Ngvtq| qrg"Gzvgpukqp" *UQNG+*83+0'Vj g"utcvgi { "hqt"vj g"uwf { "ku" vq"gzco kpg"y j gvj gt"eqpvkpqwu"mly "vto "cpvj qto qpg"vj gter { "ku'dgwt"qt"y qtug"htq"vj g" cf lwxcpv"vto gpv'qh'GT/r qukkxg"dtgcu'ecpegt"vj cp"vj gter { "vj cv'j cu"vj tgg"o qpj u'r gt" { gct" f twi "j qrkf c{ u." y j gtg" vj g" y qo gp's" qy p" gwtqi gp" ecp" f gwtq { "vj g" cpvj qto qpg" tgukucpv'dtgcu'ecpegt"egmu'dghqtg" f twi "tgukucpeg" f kugcug"i gu'c"j qrf 0"

5+Á Vj g"tgegpv"r wdrukj gf "hpf kpi u" qh" vj g" Y omen'u" J gcmj " kpkcvxg" *Y J K' qh" gwtqi gp" tgr nrego gpv'vj gter { "kp"j { vgtgevo k gf "r quwo gpqr cwucn'y qo gp"uj qy gf "c"tgf wvqp"kp" vj g"kpel' gpeg"qh'dtgcu'ecpegt"vj cv'kp"hev'eqpvkpqwu"htq"hxg" { gctu'chgt" gwtqi gp"vj gter { " uqr u"*3."4+0'Y g"ctg"r tqxkf kpi "cm'qh"vj g"uekpvhle"mpqy rgi g" f cvdcug"vq"gzr nkp"vj ku" cr r ctgpn { "r ctcf qzlecn'hpf kpi "gwtqi gp"tgr nrego gpv'tgf wegu"vj g'tkum'qh'dtgcu'ecpegt#0' Y g"qdxkqun { "veng"xgt { "ugtqun { "vj g'hev'vj cv'y g"ctg"vj g'r kpggtkpi "i tqw"uekpvhlecn { " kp"vj ku'ctgc"cpf "vj tqwi j "vj g'lpxguo gpv'qh'vj g'F QF "EqG"i tcpv'xlc"vj gkt"xkukpct { "r ggt/ tgxlgy gf "u{ ugo ." y g"j cxg" dggp"i kxgp"vj g"tgur qpukdkkv{ "vq" f gekr j gt" vj g" o gej cpkuo u" lpxqrkgf "kp"vj ku'pgy "dkqmi { "qh'gwtqi gp/kpf wegf "cr qr vuku'kp"dtgcu'ecpegt0'

K'ku'engct "htqo "vj g"chqtgo gpvqpfgf "vj tgg"dtqcf "cr r necvqp"kp"enplecn'o gf lekpg"vj cv'y g" j cxg" cp" qr r qtwpkv{ "vq" tgxqnwkpkl g" y qo gn'u" j gcmj " vj tqwi j " vj g" r twf gpv" cr r necvqp" qh" tgo clkpi "tguwtegu"cy ctf gf "vj tqwi j "qwt"EqG"i tcpv'vq"dg"vugf "kp"qwt"pq"equv'gzvgpukqp0'Ky km' u{ ugo cvlecn { "etgcvg"cp"gzgewkxg"uwo o ct { "eqpenwukqp"htq"gej "qh'qwt"qpi qkpi "Vcum0"

Vj g"r tqr qugf "enplecn'vlecn" *Vcunl3+ "j cxg"uwhtgtgf "htqo "vj g"i gpgtcn'y kj f tcy cn'qh"vj g" r j cto cegwlecn' kpf wut { " htqo " wplxgtukv{ " uekpvhle/dcu'gf " r tqi tco u0' F gur kv" vj ku" cr r ctgpn' ugdcem" y g"j cxg" o qxgf "vj g"eqqtf kpcvpi "egvgt"htqo "HEEE"vq"NEEE."cu"Kj cxg"ces wktgf " hwpf kpi "vq" o clpvclp"r cvkpv'ceetwcn'vj tqwi j "vj g"enplecn'RK" F t0'Erwlf kpg" Kucceu0' Cm' qh" vj g" dkqmi lecn'o cvgtknu"eqmgevfg "cv'HEEE"ctg"lpxcnwdng"cpf "ctg"dgkpi "vcpuhgttgf "vq"NEEE"htq" gxcnecvqp."cpn { uku"cpf "f cv" o klpki 0' K'ku"qwt"kpvgpvqp"vq" o clpvclp"r cvkpv'ceetwcn'y kj "qwt" r tko ct { "qdlgevkg"qh'uco r ng"ces wkkvqp"cpf "vj g'Epvgf "F kgevgt"cv'NEEE."F t0'Nqwu"Y gkpgt."j cu" eqo o kvgf "kpukwvqp" hwpf u"vq"r c { "htq"r cvkpv'equu0'Qwt"qtki kpcn'eqpegr v'ku'crtgcf { "kp"vj g"enplecn' f qo clp"cpf "cxckndng"htq"cm'enplekcpu"vq"vug"kp"vj gkt"vto gpv' r npu0'Vj ku'f go qpuctvgu"c"engct" uweegu'uqtf { "htq"qwt"eqo o ko gpv'vq"vj ku'tcpur'vqpnc'tgugctej 0"

Qwt"o clqt"ceeqo r rukj o gpv'vq" f cvg"qp"vj g"i tcpv'ku"vq"etgcvg"o cr "qh'vj g"rkp"cpf "f gcj "qh" dtgcu'ecpegt"egmu'kp"tgur qpug"vq"r j { ukqmi lecn'gwtqi gp"j cu"dggp"cej kxgf " *Vcunl4c+0'Vj ku'ku"c"

pgy "wpls wg" f cxcugv"j cv"ku"lpxcncdng. "dw"j g"eqo r ngzkv"qh"qwt" f cxcugv"ku"ewttgpwv" c"ej cmgpi g" vq"j g"dgudtclpu"lp"dlqkphqto ckleu"lp"j g"y qtrf. "y kj "y j qo "y g"ctg"ewttgpwv" eqmcdqtcvki 0'K'ku" ko r qtwcpv"vq"tgcrl g"j cv"qwt"xlukpct { "cr r tqcej "r tqr qugf "j g"gs wxcngpv"qh"etgcvki "c"o qxlk. "qh" j g"rlhg"cpf "f gcj "qh"dtgcuvepegt"egm"j tqwi j "i gpg"cevxckvqp"cpf "uwr r tguukqp. "dw"gxgt { "qj gt" tgugetej "i tqwr "lp"j g"y qtrf "ku"uwf { lpi "qpn { "ukpi ng"r j qvqi tcr j u"qh"egm"cpf "wo qtu"cv" c"ukpi ng" r qlpv"lp" vko g0'P gxgtj grguu. "k"ku"qwt" ceeqo r rkuj o gpv"j cv"j cu"dggp"gpj cpegf "d { "eqpukf gtcdng" dlqkphqto ckleu"lpr w"cpf "j g" f gxgr o gpv"qh"pgy "eqo r wgt"o qf grkpi "u { ugo u"vq"cpn { g"i gpg" f qukpi "cevxckvqp"ci clpuv"vko g"lqt"j g"i tqy j "cpf "f gcj "qh"j wo cp"dtgcuvepegt"egm"lp"tgr qpug" vq" gultqi gp0'Y g"j cxg"vcngp"cm"qh"qwt" gpqto qwu"i gpg"cttc { "f cv"ci clpuv"vko g"*, 8"j qwtu"cpf " r tqxkf gf "k"vq" f t0'Lqg"l tc { "cv"j g" Wplxgtukv { "qh"Qtgi qp0J g"y km'dg"y qtnkpi "y kj "wu"qxgt"j g"pgzv { gct"qh"j g"i tcpv"vq"etgcvg"cp" wpls wg"r cvj y c { "cpn { uku"oo qxlk00'Vj ku"j cu"pgxgt"dggp" f qpg"dghqtg0' Y kj "o { "grgevkp" vq"j g" P cvkpcn" Cecf go { "qh" Uekgegu"cpf "o { "lpf wevkp" f wtkpi "j g" ruv" tgr qt vki "r gtlkf. "y g"y g"j cxg"r wdkuj gf "qwt"r kpggtkpi "y qtnkpi"j g" Rtqeggf kpi u"qh"j g" P cvkpcn" Cecf go { "qh"j g" Uekgegu" *RPCU=ugg"%0"lp"Cr r gpf kz +0'Y kj "qwt" f cxcug. "y g"j cxg"ctgcf { " kf gpvklgf "j g"ugs wpeg"qh"gxgpw"lqt" gultqi gp/lpf wegf "cr qr vuku"lp"qwt" gpf qetkpg"tgukvcpv"dtgcuvepegt" egm0' Gultqi gp" lpf wegu" c" utguu" tgr qpug" cpf "cevxckv" lphco o cvqt { "i gpgu0' Vj ku" f kexgt { "pqy "cmjy u"wu"vq"lpvgtqi cvg"j ku"o gej cpluo "qh"lphco o cvkq/o gf kvgf "egm" f gcj " j tqwi j "ku"o qf wcvkq"y kj "cpvklphco o cvqt { "ci gpw"uwej "cu"i mdeqetvklf u0'Vj g"qj gt"o clqt" hpf kpi "lqo "qwt" f cxcug"ku" c" f guetk vqp"qh"j g"ecur cug"ecuecf g"j cv"r tqxqngu"egm" f gcj "cpf " f gultwevkp" hmqjy kpi " gultqi gp/lpf wegf "cr qr vuku0' Y g"j cxg"r tgeku { " f ghkpg" cpf "kf gpvklgf " ecur cug"6"cu"j g"vki i gt"ecur cug"lp"j g"lpklcvkq"qh" gultqi gp/lpf wegf "cr qr vuku0J qy gxgt. "y g"pqy " uggm"vq"dwkf "wr qp"qwt" f cxcug"cpf "wug"o qrgewr"r j cto ceqmi { "vq" f ghkpg"cpf "tghkpg"j g"lpr w" uki pcn" j tqwi j " j g" gultqi gp" tgegr vqt" j cv" o qf wcvgu" gultqi gp/lpf wegf "cr qr vuku0' Y g" ctg" cff tguukpi "j g"kuwg"qh"j y j cv"ctg"j g"dcuke" gultqi gp/GT" tgrvgf "gxgpw"j cv" vki i gt" gultqi gp/ lpf wegf "cr qr vukuA"

Y g"j cxg" hqewgf "cvgpvkp"qp"wpf gtucpf kpi "j g"tqrq"qh"e/Ute"lp"dtgcuvepegt"cpf "j g" r qvvpkn"tqrq"qh"e/Ute"lpj kdkqtu"cu"j gtr gwle"ci gpw"lqt"o gvcucvle"dtgcuvepegt0'Y g"j cxg" gxr cpf gf "qwt"y qtnkpi"j g" f kexgt { "j cv"d { "dmekpi "e/Ute."qpg"ecp"dmek" gultqi gp/lpf wegf "cr qr vuku"d { "wukpi "c"r cpn"qh"dtgcuvepegt"egm"vq"uwf { "j g"i gpgtci"cevkpu"qh" c"e/Ute"lpj kdkqt" lp"dtgcuvepegt0'Qwt"y qtnkpi"vq"dg"r wdkuj gf "lp"j g" Gwtqr gcp" Lqwtpcn"qh"Ecpegt"lp"j g"pgzv"hy " o qpjy u"*ugg"%0: "lp"j g"Cr r gpf kz "lqt"j g"r tqqlu"0'Y g"kf gpvkh { "v"r gu"qh"dtgcuvepegt"pqv"vq"dg" vgcvgf "y kj "e/Ute"lpj kdkqtu" *Vcuni4d/3+0'

Y g"j cxg"cff tguugf "j g"j { r qjy guku"j cv"d { "dmekpi "egmwrt"uwtxkcn"uki pcnki. "y g"uj qwf " dg"cdng"vq"gpj cpeg" gultqi gp/lpf wegf "cr qr vuku" *Vcuni4d/4+0'Vj g"e/Ute"qpeqi gpg"ku"r tguvpv"lp" 92" "qh"dtgcuvepegtu"cpf "ku"engctn { "c"uwtxkcn"r cvj y c { "qh"r qvvpkn"ko r qtwpeg0'Y g"j cxg"o cf g" j g"pqxgnf kexgt { "j cv"dmekpi "e/Ute"cewcm { "dmek" gultqi gp/lpf wegf "cr qr vuku0'Vj ku"eqwvgt/ lpwklxg"qdugtxcvkp"j cv"ku" wpls wg"vq"qwt"ncdqtcvt { "j cu"vq "ko r qtwcpv"tco khecvkpu<" 3+"Enplecm { "cxckcdng"e/Ute"lpj kdkqtu"uj qwf "pqv"dg" wugf "qt" vugvf "lp"dtgcuvepegt"r cvkpvu" hmqjy kpi "ftwi "tgukvpeg"vq"cpvj qto qpgu0' Vj g"e/Ute"lpj kdkqt"j cu"j g"r qvvpkn"vq"r tngxgpv" pcwtcm { "qewtltkpi " gultqi gp/lpf wegf "cr qr vuku"cpf "j ku"y km'dg"qh" f gtlko gpv"vq"j g"r cvkpv0"

4+"Qw"qdugtxcvkqp"vj cv'dmnenf g'qh'e/Ute"j cu'r qvgpvkcn'vq"lpj kdk'gustqi gp/lpf wegf "cr qr vquku"j cu' tguwngf "kp"ceqmdqtcvkqp"y kj "qwt"Ucpcf "W"4"Ecpegt"UW4E+"i tcpv'vq"gxcnxcvg"vj g"o qngewrct " o gejcpluo "vj cv'y cu'r tgxkqwnf "wmpqy p0'K'ku"ko r qtcvpv'vq"go r j cuk g"vj cv'y ku'i tcpv'cv'UW4E" j cu'pqy "lpvtqf wegf "wu"vq"ci tqwr "qh'vj g"o quv'r tqo kpgpv'cpcnf vkecn'gizr gtvu'cpf "y qtrf "ngcf gtu"lp" vj g"o qngewrct"cur gewu"qh'dtgcuv'ecpegt"lp"vj g"y qtrf 0'Vj ku'y qwf "pqv'j cxg"j cr r gpgf "dw'hqt"vj g" kpxguo gpv'lp"qwt"EQF "EqG"i tcpv0'Qw"eqmdqtcvkqp"y kj "Lqg"i tc{ 's i tqwr "j cu'tguwngf "lp"vj g" eqo r ngvkqp"qh'cp"TP Cugs "cpcnf uku"vq"kf gpvkh{ "vj g"etkkecn'tqng"qh"CR/3"*e/Hqu'cpf "e/Lxp+"vj cv'ku" kpkxcvgf "cu"ci tki i gt "qh'cr qr vquku"46"j qwtu'chgt "gustqi gp"ko r cewu"qp"vj g"egm0'Y g"j cxg"r tqxgf " vj cv'egm'ecp"dg'tguewgf "wr "vq"46"j qwtu'htqo "gustqi gp/lpf wegf "cr qr vquku"0'Y g"j cxg"r go qpvtcvgf " cpf "y km'eqpvkpwg"v wtkpi "qwt"pq"equv'gzvgpukqp."vj g"etkkecn'tqng"qh"CR/3"uki pcnki "lp"cr qr vquku' Vj tqwi j "c"eqmdqtcvkqp"y kj "Ft0'O {ngu"Dtqy p"cv"J ctxctf "Wpksgtukv{."y g"j cxg"kf gpvkhgf " ej tqo cvkpd'lpf kpi "ukgu"lp"qpg"qh'qwt"egm'hpgu"vj cv'ku'ctqo cvcug'tgukxcpv."OE/9-4C"84+"ugg"% 35"lp"vj g"Cr r gpf kz+0'Y g"ctg"ewtgpvnf "f gvgto klpki "GT"vcti gu'lp"qwt"OE/9-7E"egm'vj cv'ecp"dg" cevxcvgf "f wtkpi "vj g"htuv'46"j qwtu'vq'etgcvg"CR/3"hwpevkpcn'wpku0'"

" Y g"dgngxg"vj cv"ci etkkecn'kuwg"vj cv"o c{"ckf "qwt"wpf gtucpf kpi "qh"gustqi gp/lpf wegf " cr qr vquku"lp"dtgcuv'ecpegt"o c{"dg"vq"fkueqxtg"y j cv'y g"o gejcpluo "qh"gustqi gp/lpf gr gpf gpv' i tqy vj "cewcmf "ku"*Vcuni'4d/5+0'Vq"cf f tguu"vj ku's wguvkqp."y g"j cxg"fkueqxtg"vj cv'eO [E"ku"ci rtko g'wtxkcn'o gejcpluo "hqt"gustqi gp"lpf gr gpf gpv'i tqy vj 0'Y g"j cxg"tki qtqwnf "gzco kpgf "vj g" tgi wrcvkqp"qh'eO [E"qp"vj g"egm'e{eng"cpf "qwt"y qtni'r tqxkf gu"ci o gejcpluo "wpf gtucpf kpi "qh" cwqpqo qwu"i tqy vj "qh"dtgcuv'ecpegt"hmny kpi "vj g"fgxgr o gpv'qh"tgukxpeg"vq"ctqo cvcug" lpj kdkqtu'0'Y g"ctg"r rcpkpi "vq"eqo r ngv"qwt"uwf lgu'f wtkpi "qwt"pq"equv'gzvgpukqp"cpf "uwo k'qwt" y qtni'hqt"r wdrkcvkqp"y kj lp"vj g"pgzv'8"o qpj u0'

Cpqj gt"ko r qtcvpv'cur gev'qh'vj g"tki i gt kpi "qh"gustqi gp/lpf wegf "cr qr vquku"ku"vj g"cewcnf uj cr g'cpf "eqphqto cvkqp"qh'vj g"GT"eqo r ngz "lp"vj g"egm'*Vcuni'4d/6+0'Gctnkt."y g"fguetkdgf "c"pgy " encuukhkecvkqp"qh"u{pvj g'ke"cpf "pcwtcn'gustqi gpu"dlpf kpi "vq"vj g"gustqi gp"tgegr vqt."cpf "vj ku' encuukhkecvkqp"tgcmf "ugi tgi cvgf "vj g"o qngewgu'lpvq'r rcpct"cpf "pqp/r rcpct"gustqi gpu0'Cu"ci tguwn'qh" rki cpf "dlpf kpi ."vj g'r rcpct"gustqi gpu"Encuu'K'r tqf wegf "c"pgcv'r tqvklp"eqo r ngz "ctqwpf "vj g'r rcpct" gustqi gp0'K'eqpv'cuv."pqp/r rcpct"gustqi gpu"Encuu'K'f q"pqv'cmqy "vj g"gustqi gp"tgegr vqt"vq"enqg" pgcvn{ "ctqwpf "vj g'rki cpf "cpf "cv'ku"gzvto g."cp"cpv'gustqi gp'r tqf wegu'cp"cdpqto cn'uj cr g."vj gtgd{ " dmnenkpi "gustqi gp"cevkqp"*47+0'Gctnkt"y g"fkueqxtg"vj cv'pqp/uvgtqkf cn'cpv'gustqi gpu"*UGTO u" uwej "cu"6/j {ftqz{wco qzkgp."eqo r ngvgnf "dmengf "gustqi gp/lpf wegf "cr qr vquku"0'Vj g"uj cr g'qh'vj g" eqo r ngz"engctn{ "y cu' etkkecn' vq"tki i gt"cr qr vquku"0'J qy gxgt."y g"j cxg"pqy "gzvgpf gf"qwt" kpxguki cvkqp"wukpi "Encuu'K'gustqi gpu"*Vcuni'4d/6'cpf '4d/7+0'Cnj qwi j "gustqi gp/rkng."Encuu'K' gustqi gpu"unko wrcvg"dtgcuv'ecpegt"egm'tgr rkecvkqp."dw'f q"pqv'lp"hecv'unko wrcvg"gustqi gp/lpf wegf " cr qr vquku"0'Vj g"tki i gt kpi "o gejcpluo "umy gf "f qy p"cpf "cr qr vquku"pqy "qeewtu'cdqww'9'f c{u'ncvt0' K'ku"ci tgo ctmedng"fgo qpvtcvkqp"qh'j qy "y g'ecp"o qf wrcvg"vj g"tki i gt"vj tqwi j "vj g'uj cr g'qh'vj g"GT" eqo r ngz0'Vj ku'lpuki j v'lpvq"vj g"o qf wrcvkqp"qh'vj g'uj cr g'qh'vj g"GT"eqo r ngz"pqy "ftcy u'wu"vq"vj g" eqpenmukqp"vj cv'eq/cevxcvqtu"vj cv'y qwf "pqto cmf "dlpf "vq"vj g"GT"eqo r ngz"htq'hwngustqi gp"cevkqp" ctg"engctn{ "etkkecn'hqt"gustqi gp/lpf wegf "cr qr vquku"0'Y g'r rcp"vq"kpkkcvg"ci o clqt"r tqlgev'uwf {kpi " vj g"utwewt g'hwpevkqp"tgncvkqpj k r u"qh'Encuu'K'gustqi gpu"cpf "vj gk"cdkdkv{ "vq"o qf wrcvg"cr qr vquku"

cpf "f grkxgt"cr r tqr tlcvg"eq/cevxcvqtu"vq"r tqo qvgt"ukgu"qp"guvqi gp"vcti gv'i gpgu"fwtkpi "vj g"pgzv
{ gct0'Qwt"r tqi tguu"lp"Vcuni4d/6'ēpf '4d/7"ku"j ki j n' "uki pñkēcpv."cu'y g"fgo qpwtcvg"vj cv'Erūu"K
guvqi gpu"unqy "f qy p"cr qr vquku"vj tqwi j "vj g"ugeqpf "y ggm'qh"guvqi gp"gzr quwtg0'Vj ku"eqpvtcuu"
y kj "r mpcet"Erūu"Kguvqi gpu"vj cv'ecwug"cr qr vquku"lp"vj g"htuv'y ggm"Vcuni4d/6+0'Y g"j cxg"cnq"
f lueqxtgf "vj cv'ej go qvj gtr { "y kj "c"ucpf ctf "dtgcu'ecpegt"ftwi . "r cerkczgn"ecwugu"ko o gf kcvg"
cr qr vquku"d { "vj g"gzvtpule"*f gcvj +"tgegr vqt "rcvj y c { "Vcuni4d/7+0'K"lp"eqpvtcuu" guvtf kqnl'kpñkcvu"
cr qr vquku"vj tqwi j "vj g"lpvtpule"rcvj y c { "lp"o kqej qpf tlc"cpf "vj gp"vj g"egm"eqo r rvgv"vj g"rtqegu"
d { "tgetwklpi "vj g"gzvtpule"rcvj y c { "Vcuni4d/7+0'K"ku"ko r qtvcpv"vq"utguu"vj cv'vj g"i qcn'qh"vj ku"
o qrgewrt" gpvtr tkug" qh"vj g"uj cr g" qh"vj g" GT" vq" o qf wrcv" guvqi gp/kpf wegf " cr qr vquku" pqy "
r gthgevn'lpvgtugev'y kj "Vcuni5."vj g"rtqvgqo leu'qh'guvqi gp/kpf wegf "cr qr vquku0"

Vj g"tqng"qh"qwt"r tqvgqo le"i tqw u"Vcuni5+"ku"vq"mqm'cv"vj g"gctn { "uci gu"qh" guvqi gp"
tgegr vqt"o gf kcvf "guvqi gp/kpf wegf "cr qr vquku"vq"fg hkp"ng { "eqo r qpgrvu"qh"vj g"GT"eqo r rnz"qt"
rcvj y c { u"go cpcvki "htqo "cp"ko r qtvcpv"pqf g0'Qwt"r tqvgqo leu"i tqw "j cu"lpvgti tcvf "c"i mdcn'eq/
cevxcvqt"uki pcnki "pgwy qtm"CD3+"vj cv'cr r gctu"vq"eqpvtqn'vj g"i tqy vj "cpf "cr qr vquku"qh"dtgcu'
ecpegt"egm0' Vj g" ko r qtvpeg" qh" I /r tqvlp" eqwr ngf " tgegr vqtu. " RK5" nkpug. " Y pv" cpf " P qvej "
uki pcnki "rcvj y c { u"ctg" utqpi n { "cuuqekcvf "y kj " guvqi gp/kpf wegf "r tqñkgtcvkp"qt" cr qr vquku0'
Vj gug"lpf lpi u"rpnlp"vq"qwt"r tkqt"r wñkcvkp"qp" I RT52"*85+"cpf "qwt"y qtm'qp"vj g"uj cr g"qh"vj g"
GT" cpf " guvqi gp/kpf wegf "cr qr vquku0' K" qwt"r ctcngn"uwf lgu. "y g"eqpenmf gf "vj cv' CD3"ku"vj g"
eqpvtqnki "o gejcpluo "vq"vki i gt"guvqi gp/kpf wegf "cr qr vquku"lp"qwt"cpv'j qto qpq"tgukncpv'dtgcu'
ecpegt"egm0'K" [gct"7"qh"qwt"i tcpv."y g"wugf "dtgcu'ecpegt"wo qtu"i tqy p"lp"cvj { o le"o leg"vq"
cftfguu"vj g"uco g"s wugkp"y g"j cxg"f gtxgf "j gtg"lp"egm'ewwtg"*62+"dw"lp"xkxq0'Y g"j cxg"
f lueqxtgf "pqxgn'i gpg'cevxcvqp"pgxgt"eqpuk'gtgf "rtgxkwun'0'Vj gug"fcv"ctg"vj g"htuv"vq"tgr qt v'
vj cv'vj g"hwpevqp"qh" RJ Du"cu"cp" GT" eq/tgr tguuqt"ecp"dg" tgi wrcv" d { "r quv'tcpurcvkp'cn'
o qf hñkcvkp"*RVO u+0'Vj g'y qtm'ku'dgkpi "r tgr ctgf "hqt"r wñkcvkp0'

Vj g"i gpqo leu'r tqi tco "cv'VI gp"Vcuni6c+"j cu'cee qo r rkuj gf "c'tgo ctmēdng"cpn { uku"qh"vj g"
tgukpi "cpf "i gpg'cevxcvqp"ucvgu"qh'qwt"cpv'j qto qpq'tgukncpv'dtgcu'ecpegt"egm'lp"ewwtg0'Cu"c"
uwr r qtvkxg"eqo o ko gpv."F t0'J gcvj gt" Ewprñhg"htqo "VI gp"j cu"eqpf wcvf "c"j ki j /vj tqwi j r w'
cpn { uku"wukpi "ukTP Cu"vq"f gvgto kp"vj g"r tgekug"i gpgu'cevxcvqf "d { "guvqi gp/kpf wegf "cr qr vquku0'
Vj gug"fcv"ctg"wpkxg"cpf "o grf "r gthgevn' "y kj "cm'qh'qwt"qvj gt'vcum0'Vj g"fcv"i gpgtcvqf "y kn'dg"
wugf "lp" [gct"8"qh"vj g"pq"equv'gz vgpukp"vq" f lueqxtgf "vj g"cr qr vqke"vki i gt"qh"vj g"guvqi gp"tgegr vqt"
eqo r rnz0'

Qwt"hwwtg'r ncpu'hqt"vj g"pq"equv'gz vgpukp"y kn'eqo r rvgv"qwt"qtki kpcn'r mpc'hqt"fgelr j gtlpi "
vj g"o qrgewrt"o gejcpluo "o gejcpluo u"qh" guvqi gp/kpf wegf "cr qr vquku0'Qwt"wpkxg"vgco "j cu"
dwkn'qp"qwt"utgpi vj u'cpf "y g"ctg'pqy "r qkugf "vq"lpvgttqi cvg"vj g"o qf gnu'cpf "o qxg'tcr kñ { "vqy ctf u"
r wñkcvkp0'Y g"ctg"vj g"ngcf gtu"lp"vj ku'ctgc0'K"ku"ko r qtvcpv"vq"utguu"vj cv'qwt"fcv"y cu"wugf "vq"
qdvcp"i tcpw"htqo "qvj gt"uqwtgu"*UW4E."Uwucp" I 0'Mqo gp"Hqt"Vj g"Ewtg+0'Vj ku"gpj cpegu"qwt"
ecr cekl { "hqt"lpvgtcvkp"y kj "vj g"dguv'dtgcu'ecpegt'tgugctej "uelgpvkuu"lp"vj g'y qtrf 0"

TGHGTGPEGU'

30' Cpf gtuqp" I N."Ej ngdqy unkTV."Ctci cnk'CM" Mwngt"NJ . "Ocpuqp"IG."I cuu'O ."Dnñj o "G."
Eqppgm { "U."J wddgm'HC."Ncp" F ."O ctvlp"N."Qengpg"L" Tqj cp"V."Uej gpngp" T."Y cevcy unk'Y gpf g"

l0'Eqplwi cvgf "gs wkpq" qgustqi gp"cpf "dtgcu"ecpegt"lpekf gpeg"cpf "o qtvrk\{ "kp"r quwo gpqr cwucn' y qo gp"y kj "j {ugtgevqo {<gzvpgf gf"lqmny /wr "qh"vj g"Y qo gp)u"J gcnj "kpkkcxg"tcpf qo kugf " r nregdq/eqptqngf "tkr0NcpegvQpeqi04234=35*7+698/6: 80'

40' Ej rgdqy unK'TV."Cpf gtupq"I N0'Ej cpi kpi "eqpegr w<O gpqr cwucn'j qto qpg"vj gter {"cpf " dtgcu"ecpegt0L'PcwiEcpegt'kpa04234=326*9+739/7490'

50' Grku'O L'I cq'H'F gj f cuj v<H'Lghg'F D."O cteqo 'RM'Ectg{"NC.'F lemgt'O P.'Ukxgto cp" R."Hgo kpi "I H"Mo o ctgff {"C."Lco cmcdf k/O clkf k'U."Etqy f gt"T."Ukgi gn'DC0'Nqy gt/f qug"xu" j k j /f qug" qtcn' gutfc kqn' vj gter {" qh"j qto qpg" tgegr vqt/r qukxg." ctqo cvcug" kpj kdkqt/tgukxcpv' cf xcpegf "dtgcu"ecpegt<c'r j cug"4'tcpf qo k gf "uwf {0LCO C0422; =524*9+996/9: 20'

60' O c {gt'GN."Dcwtkp'lh"Ur ctepq'L"Utcwuu"N."Eco r qpg'O."Hwo qrgcw'R."Twi q'J . "Cy cf c" C."U{"Q."Nqo dctvEwuue"C0'C"r j cug"4"tkrnf qh'f cucvklp"kp"r cvkpw"y kj "cf xcpegf "J GT4/ r qukxg"cpf lqt"j qto qpg"tgegr vqt/r qukxg"dtgcu"ecpegt0'Erkp"Ecpegt"Tgu04233=39*43+8: ; 9/ 8; 260'

70' Urco qp"FL"Ertm'I O."Y qpi "UI ."Ngxlp"Y L"Whtlej "C."O eI vktg"Y N0'J wo cp"dtgcu" ecpegt<eqttgrvklp"qh"tgrug"cpf "uwxkcn"y kj "co r nhecvklp"qh"vj g"J GT/4 lpgw" qpeqi gpg0' Uekpeg03; : 9=457*69: 7+399/3: 40'

80' Vj qo cu"UO."Dtwi i g"LU'Egmwrt"hwpevklpu"tgi wvvgf "d{"Ute"lco kn{"mkpcugu0'Cppw'Tgx" Egm'F gx'Dkqr03; ; 9=35<735/82; 0'

90' Uj wr plm'O C0'Etquucm'dgwy ggp"ugtqkf "tgegr vqtu"cpf "vj g'e/Ute/tgegr vqt"v\ tqukpg"mkpcug" r cvj y c {u<lo r nhecvklpu"ht"egmr tqnhtcvklp0'Qpeqi gpg04226=45*6: +9; 9; /9; : ; 0'

: 0' Ngy ku'LU."O ggng'M"Quk q'E."Tquu'GC."Mf cy k'P."Nk'V."Dgm'G."Ej cpf grnP U."Lqtf cp"XE0' kptkpule"o gej cpkuo "qh"guvtf kqn'lpf wegf "cr qr vuku"kp"dtgcu"ecpegt"egm'tgukxcpv"vq"guvtf gp" f gr tkxcvklp0L'PcwiEcpegt'kpa04227=; 9*45+3968/397; 0'

; 0' Ctlcl k'GC."Ewprkthg"J G."Ngy ku/Y co dk'LU."Urhngt"O L"Y knku"CN."Tco qu"R."Vcr kc"E." Mko "J T."l gttwo "U."Uj cto c'EI ."P leqrnu"G."Dcmi wvpcvj cp"l ."Tquu'GC."Lqtf cp"XE0'Guvtqi gp" kpf wegu"cr qr vuku"kp"guvtf gp" f gr tkxcvklp/tgukxcpv"dtgcu"ecpegt"vj tqwi j "utguu'tgur qpugu"cu" kf gpvklgf " d{" i nqden' i gpg" gzrtguukqp" cetquu" vko g0' Rtqe" Pcw' Cecf" Uek' W" U' C0' 4233= 32: *69+3: : 9; /3: : : 80'

320' O cuwfc"U."Wo gfc"O."Wej kfc"J ."Mcvq"J ."Ctenk' V0' Cngtcvklpu"qh"qz kfcvkg"utguu" o ctngtu"cpf "cr qr vuku"o ctngtu"lp"vj g"utkwo "chgt"tcpukgpv'hqecn'egtgdten'kuej go kc"kp"tcwu0L" Pgwtn'Vtcpuo 0422; =338*6+5; 7/6260'

330' Co g{ct'O."Y kuplgy unc'O."Y gkl o cp'LD0C'tqng'ht"CR/3'lp"cr qr vuku<vj g'ecug'ht"cpf " ci ckpu0'Dkqej ko kg04225=; 7*: +969/9740'

340' Uej kth" T."Tgff {"R."Cj qwr c"O."Eqtpcf q/J glpuqj p" G."I tko "O."J knugdgem' UI ." Ncy tgepg" T."F gpgng"U."J gttgtc" T."Ej co pgui" I E."Hws wc" UC."Dtqy p" RJ ." Qudqtpg" EM0' Qz kfcvkg"utguu"cpf "CR/3'cevklv\{ "kp"vco qz khp/tgukxcpv"dtgcu"wo qtu"lp"xlxq0L'PcwiEcpegt" kpa04222=; 4*45+3; 48/3; 560'

350' Co g{ct/\ c| qwc'O."Y kuplgy unc'O D."Denk'k'N."Y ci pgt"GH"l cplx'O."Y gkl o cp'LD0CR/ 3"f ko gtu"tgi wvvg"vcpuetkr vklp"qh"vj g"r 36lr 3; CTH'wo qt"uw r tguuqt"i gpg0' Qpeqi gpg04227= 46*36+44; : /45280'

360' \ j cpi "Z." \ j cpi "N." [cpi "J ." J wcpj "Z." Qw"J ." Nldgto cpp"VC." F gY qrh"Y E." Mj qutcxk/ Hct" T." Qmwo k'CH0e/Hqu"cu" c"r tqcr qr vqve"ci gpv"lp"VTC KN/ kpf wegf "cr qr vquku"lp"r tqwcvg"ecpegt" egm0Ecpegt "Tgi04229=89*3; +< 647/; 6560'

370' I qpi "R." Ugy ctv" F." J w" D." Xlpuqp" E." Cmo "L0' O wnr ng" dcule/ngwelpg" | kr r gt" r tqvgkpu" tgi wrwv" kpf wekqp" qh" yj g" o qwug" j go g" qz { i gpcug/3" i gpg" d { "ctugpkv0' Ctej "Dkvej go "Dkqrj { u0' 4224=627*4+487/4960'

380' O kngt" VY ." Dcmq" LO ." I j c| qwk" \ ." F wpdkg" C." Cpf gtup" J ." F qy ugw" O ." I qp| cmg| / Cpi wmq" CO ." O km" I D." O kngt" Y T." Y w" J ." Uj { t" [." Ctvgci c" EN0' C" i gpg" gzt tguukqp" uki pcwtg" htqo " j wo cp" dtgcu" ecpegt" egm" y kj " ces wktgf " j qto qpg" kpf gr gpf gpeg" kf gpvhlgu" O [E" cu" c" o gf kvqt" qh'cpvkgutqi gp" tguukpeg0' Enkp" Ecpegt "Tgi04233=39*9+4246/42560'

390' Y qrhgt" C." Y kwpgt" DU." Kko kc" F." Hrcxlp" TL" Nwr kgp" O ." I wpcy ctf cpg" TP ." O g { gt" EC ." Nki j vecr "GU." Vco c { q" R." O guktqx" IR." Nkw" ZU." Uj kqf c" V." Vqpgt" O ." Nqf c" O ." Dtqy p" O ." Dtwi i g" LU." Tco cuy co { "U0O [E" tgi wrwv" qh'c" \$r qqt/ r tqi pquku\$ " o gcuwvle" ecpegt" egm'ucv0' Rtqe" Pcvl' Cecf" UekW" UC04232=329* . +58; : /59250'

3: 0' Nlcq" FL" F lemuqp" TD0e/ O { e" lp" dtgcu" ecpegt0' Gpf qet "Tgrv" Ecpegt 04222=9*5+365/3860'

3; 0' O g { gt" P ." Rgpp" N\ 0Tghngevki "qp" 47" { gctu" y kj " O [E0' Pcv" Tgx" Ecpegt 0422: =: *34+< 98/ ; ; 20'

420' Uj cpi "I ." Dtqy p" O 0O qrgewrt "f gvgto kpcpu" hqt " yj g" kuwv" ur gekhkv { "qh" UGTO u0' Uekpeg0' 4224=4; 7*7786+4687/468: 0'

430' Uj cpi "I ." J w" Z." F kT gp| q" L" Nc| ct" O C." Dtqy p" O 0' Eqhcevt "f { pco leu" cpf "uwthlekgpe { "lp" gutqi gp" tgegr vqt/ tgi wrwv" t'cpuetkr vqp0' Egm04222=325*8< 65/ : 740'

440' O cti ctkku" V." J qnvgi g" HE0' Rqlugf "TP C" r qn { o gtcug" KK' i kxgu" r cwug" hqt " yj qwi j w0' Egm0' 422: =355*6+7: 3/7: 60'

450' Ucwpf gtu" C." Eqtg" NL" Nku" LV0' Dtgcnkpi "dcttkgu" vq" t'cpuetkr vqp" gnupi cvkqp0' Pcv" Tgx" O qn' Egm' Dkqr04228=9* . +779/7890'

460' Ugpi wr w" U." Lqtf cp" XE0' Ugrgevxg" gutqi gp" o qf wrwvtu" cu" cp" cpvlecpegt "vqqr" o gej cpkuo u" qh'ghlekgpe { "cpf "tguukpeg0' Cf x" Gzr " O gf " Dkqr0422: =852-428/43; 0'

470' Lqtf cp" XE." Uej chgt" LO ." Ngxgpuqp" CU." Nkw" J ." Rgcug" MO ." Uko qpu" NC ." \ cr h' LY 0' O qrgewrt "ercuuklec vqp" qh' gutqi gpi0' Ecpegt "Tgi04223=83*3: +883; /88450'

480' Lqtf cp" XE." QO cmg { " DY 0' Ugrgevxg" gutqi gp/ tgegr vqt " o qf wrwvtu" cpf " cpvj qto qpcri' tguukpeg" lp" dtgcu" ecpegt0' L' Enkp" Qpeqr04229=47*58+7: 37/7: 460'

490' Dt| q| qy unk" CO ." Rkng" CE." F cwgt" \ ." J wddctf "TG." Dqpp" V." Gpi utqo " Q." Qj o cp" N." I tggpg" I N." I wuchuqp" LC." Ectrs wku" O 0' O qrgewrt "dcuku" qh' ci qpkuo " cpf " cpvci qpkuo " lp" yj g" qgutqi gp" tgegr vqt0' Pcvwt g03; ; 9=5: ; *8874+975/97: 0'

4: 0' Uj kw" CM." Dcturf " F." Nqtke" RO ." Ej gpi " N." Mwuj pgt" RL " Ci ctf " F C." I tggpg" I N0' Vj g" utwewtcri' dcuku" qh' gutqi gp" tgegr vqt leqcevxvqt " tgeqi pklqp" cpf " yj g" cpvci qpkuo " qh' yj ku" kpgtcevkvqp" d { " vco qz hgp0' Egm03; ; : =; 7*9+< 49/; 590'

4; 0' Ngxgpuqp" CU." Ecvj gtlpq" Y J ." Lqtf cp" XE0' Gutqi gple" cevkvk { " ku" kpetgcugf " hqt" cp" cpvkvutqi gp" d { " c" pcwtcri' o wcvkqp" qh' yj g" gutqi gp" tgegr vqt0' L" Ugtqlf " Dkvej go " O qn' Dkqr03; ; 9= 82*7/8+483/48: 0'

520' Ngxgpuqp"CU."Vqpgwk'F C."Lqtf cp"XE0'Vj g"qgustqi gp/rkng"ghhev'qh'6/j {ftqz{vco qzkhep" qp"lpf wevkqp"qh'vcpuhqto kpi "i tqy vj "hcevt"crj c"o TPC"kp"OFC/OD/453"dtgcu'vecepgt"egmu" uedn{ "gzt tguukpi "vj g"qgustqi gp'tgegr vqt0Dt'LEcpegt03; ; : =99*33+3: 34/3: 3; 0'

530' Y qh'FO."Lqtf cp"XE0'Vj g"ustqi gp"tgegr vqt"ltqo "c"vco qzkhep"unko wvvgf"OEH/9"wo qt" xctkcpv'eqpvckpu"cr r qkp'o wcvkqp"kp"vj g"rki cpf"dlpf kpi "f qo ckp0Dt'gcu'Ecpegt'Tgu'Vtgc'03; ; 6= 53*3+34; /35: 0'

540' O cel tgi qt"Uej chgt"L"Nkw"J ."Dgptgo "FL"\ cr h'LY ."Lqtf cp"XE0'Cmugtle"ukgpekpi "qh' cevxcvki "hpevkqp"3"kp"vj g"6/j {ftqz{vco qzkhep"ustqi gp"tgegr vqt"eqo r rnz"ku"lpf wegf"d{" uwdurkwkpi "i n{ekpg'hqt'curctwv'cv'co kpq'cekf'5730Ecpegt'Tgu'04222=82*3: +72; 9/73270'

550' Dgptgo "F."Hqz"LG."Rgcteg"UV."Nkw"J ."Rcr r cu"U."Mw hgt"F." cr h'LY ."Lqtf cp"XE0' Fkukpev"o qrgewrt"eqphqto cvkpu"qh"vj g"ustqi gp"tgegr vqt" crj c"eqo r rnz" gzt rnkvgf"d{" gpvktqpo gpvni'ustqi gpu'0Ecpegt'Tgu'04225=85*43+96; 2/96; 80'

560' O czko qx"Rl ."O {gtu'ED."Ewtr cp"TH"Ngv ku/Y co dk'LU."Lqtf cp"XE0'Utwewtg/hpevkqp" tgrcvkpuj kr u"qh'ustqi gpke"vkrj gp{rvj {rgpgu'tgrvvgf"v"gpqzkhgp"cpf"6/j {ftqz{vco qzkhep'0L" O gf'Ej go 04232=75*: +5495/54: 50'

570' J cff qy"C."Y cvnkpuqp"LO."Rcvtuqp"G."Mqngt'RE0'Khmvgpeg"qh'U{pvj gve"Qgustqi gpu"qp" Cfxcpegf'O crki pcpv'F kugcug'0Dt'O gf'LO3; 66=4*658: +5; 5/5; : 0'

580' Nqppkpi "RG."Vc{rqt"RF."Cpngt"I ."Kf qp"L"Y kg"N."Lqti gpugp"NO."O gmc"Q."J qy gm'C0' J ki j /f qug"ustqi gp" vgcwo gpv'kp"r quvo gpqr cwuci' dtgcu' ecpegt"r cvkpgpu"j gcxkn{ "gzt qugf"v" gpqf qetkpg"vj gtr {0Dt'gcu'Ecpegt'Tgu'Vtgc'04223=89*4+333/3380'

590' NcEtqkz'C\ ."Ej rgdqy unk'TV."Ocpuqp"LG."Ctei cnk'CM."Lqj puqp"ME."O ctv'N."O cti qru" MN."Ughcpkeni'ON."Dt{ unk'T."Ewtd'LF."J qy ctf"DX."Ngv ku"EG."Y cevcy unk'Y gpfg'LO'J gcnj " qweqo gu'chgt"uvr r kpi "eqplwi cvgf"gs wkp"ustqi gpu'co qpi "r quvo gpqr cwuci'y qo gp'y kj "r tkqt" j {uytgevqo {<c'tcpf qo k gf"eqpvqmgf"vkrn'0LCO C04233=527*35+3527/35360' 5: 0' [cq'M'Ngg'GU."Dgptgo "FL"Gpi rcpf'I ."Uej chgt'LK'Q'Jgi cp"TO."Lqtf cp"XE0'Cpvkwo qt" cevkvq"qh'rj {ukvni kecn'ustcf kqn'qp"vco qzkhep/unko wvvgf"dtgcu'wo qtu'i tqy p'kp"cvj {o le"o keg'0' Erkp'Ecpegt'Tgu'04222=8*7+424: /42580' 5; 0' S kp"l [."NKJ."I vq'ZL"l g'ZH"Y gkZ."j qw"J ."j cpi "ZL"Y cpi "E."S kp"Y ."NwL"J g" LO'Cflwvcpv'ej go qv gtr {."y kj "qt"y kj qw'vzcpgu."kp"gtcn{ "qt"qr gtcdrng"dtgcu'ecpegt<c"o gvc/ cpcn{uku'qh'3; 'tcpf qo k gf"vkrn'y kj "528; : 'r cvkpgpu'0RNqUQpg04233=8*33+g48; 680'

620' J w\ \ ."Mci cp"DN."Ctkl k'GC."Tqugvj cn'FU."j cpi "N."Nk'LY."J wpi "J ."Y w'E."Lqtf cp" XE."Tkgi gn'CV."Y gmvgkp"C0'Rtqvqo le"cpn{uku'qh"r cvj y c{u"kpqxkvgf"kp"ustqi gp/lpf wegf" i tqy vj "cpf"cr qr vuku'qh'dtgcu'ecpegt'egmu'0RNqUQpg04233=8*8+g426320'

630' J g"D."Hgi "S."O wnj gtgg'C."Nqptcf "FO."F go c{q"HL"Mc'v gpvgmpdqi gp"DU."N{f qp'IR." QO cmg{ "DY 0'C"tgr tguukg"tqng'hqt"r tqj kdkkp"kp"ustqi gp"uki pcrkpi 0'O qn'Gpf qetkpg'0'422: = 44*4+566/5820'

640' F qo cpunk'F."O wtrj {"NE."Dqtej gtu'EJ 0'Cuuc{"f gxgnr o gpv'hqt"vj g"fgvto kpcvkqp"qh" rj qurj qt{rvkvq"uvkej kqo gt{"wukpi "o wmr ng"tgcevkvq"o qpkqtkpi "o gvj qf u'y kj "cpf"y kj qw" rj qurj cvcug" vgcwo gpv' cr r rkevkvq"v"dtgcu' ecpegt"uki pcrkpi "r cvj y c{u'0' Cpcn'Ej go 0' 4232= : 4*35+7832/78420'

650' Twug"EK"Y kmtf "D."Lp"LR."J ccu"V."Mkpgt"O."Dqpf"O 0'S wcpkckxg"f {pco leu"qh'ukg/
ur gekle'r tqvgk"r j qur j qt {rvkqp"f gvgto kpgf "wukpi "rks wlf"ej tqo cvqi tcr j { "grgestqur tc { "kqpk cvkqp"
o cuu'ur gestqo gxt {0Cpcn'Ej go 04224=96*9+387: /38860'

660' Co cpej { "T."Rgtkuy co { "D."O cvj kxcpcp"U."Tgf f { "T."Vcwknqvc"UI ."Rcpf g { "C0C"ewtcvgf "
eqo r gpf kwo "qh'r j qur j qt {rvkqp"o qvhu0PcvDkqvgej pqr04229=47*5+4: 7/4: 80'

670' Dnqo "P."Ulej gtl /Rqpvgp"V."I wr c" T."I co o gnqhv"U."Dtwpcn' U0 Rtgf kvkqp"qh"r quv/
xcpurvkqpcn"i n{equ{rvkqp" cpf " r j qur j qt {rvkqp" qh" r tqvgkpu" htqo " y j g" co kpq" cekf " ugs wgpeg0'
Rtqvqgo keu04226=6*8+3855/386; 0'

680' Ngy ku/Y co dk'LU."Lqtf cp"XŒ0'Gutqi gp"tgi wrvkqp"qh"cr qr vuku<j qy "ecp"qpg"j qto qpg"
unko wrvg"cpf "lpj kdkvA'Dtgcw/Ecpegt "Tgu0422; =33*5+4280'

690' P kvo cpu"NI ."Ctvcn'UO ."I tlxgm'NC."Eqcvgu"RL0Vj g"o kqej qpf tkcn'RJ D"eqo r ngz<tqrgu"
kp"o kqej qpf tkcn'tgur kcvqt { "eqo r ngz "cuugo dn{ ."ci gkpi "cpf "f gi gpgtcvkxg"f kugcug0'Egm'O qn'Nkg"
Ue04224=7; *3+365/3770'

6: 0' Telcnkpi co " M" Twf gn' V0' Tcu/Tch" uki pcnkpi " pggf u" r tqj kdkkp0' Egm' E{erg0' 4227=
6*33+3725/37270'

6; 0' Telcnkpi co "M"Y wpf gt"E."Dtkpno cpp"X."Ej wtkp"[."J gno cp"O."Ukxgtu"E."Tcr r "WT."
Twf gn' V0'Rtqj kdkkp"ku"tgs wktgf "hqt" Tcu/kpf wegf "Tch/O GM/GTM"cevxcvkqp"cpf "gr kj grkcn'egm'
o ki tcvkqp0PcvEgm'Dkqr04227=9*: < 59/: 650'

720' Ocnkuj ko c" V." [quj ko k' O."Mqo k{co c" U." J etc" P." P kuj ko qvq" V0' C" uwdwpx" qh" y j g"
o co o cnkcp"qrki quceej ct {ntcpuhgtcug."F CF 3."kpgtcew"y kj "O en/3."qpg"qh" y j g"den/4" r tqvgkp"
hco kn{0L'Dkqej go 04222=34: *5+5; ; /6270'

730' Vct| g"C."F gpkwlf "C."Ng"Dtcu"O."O cknkt "G."O qmg"F."Nctqej gwg"P." \ co | co kP."Lcp" I ." "
Mtqgo gt" I ."Dtpppgt"E0I CRF J ."c"pqxgnltgi wrvqt"qh'y j g'r tq/cr qr vqle"o kqej qpf tkcn'o go dtcpg"
r gto gcdknk cvkqp0Qpeqi gpg04229=48*3: +4828/48420'

740' Tk| wq" T."F wej gp"O T."Rq| | cp"V0'Hktvpi "kp"rkvwg"ur ceg<y j g"GT lo kqej qpf tkc"Ec4- "
rckuqp0Uek'UMG04226=4226*437+tg30'

750' Dtqy o cp"F V."J qgi i "O D."Tqddkpu"UO 0'Vj g"URHJ "f qo ckp/eqpvcklpi "r tqvgkpu<o qtg"
y j cp"rk kf "tchn'o ctngtu0Vt gpf u'Egm'Dkqr04229=39*: +5; 6/6240'

760' Dtqy o cp"F V."Tgugm'O G." \ clej qy unk'NF."Tqddkpu"UO 0'Gtnk/3"cpf "gtnk/4"ctg"pqxgn"
o go dgtu"qh'y j g'r tqj kdkkp" hco kn{ "qh'r tqvgkpu"y j cvf ghkpg"rk kf /tch/rkng" f qo ckpu"qh'y j g"GT0L'Egm"
Ue04228=33; *Rv37+536; /53820'

770' Cpf g"UT."I w[."P {qo dc"DN."O kuj tc"U0Kpuwkp"lpf wegf "r j qur j qt {rvkqp"qh'r tqj kdkkp"cv"
v{tqukpg"336'tgetvku"Uj r 30Dkqej ko 'Dkqr j {u'Cew0422; =39; 5*: +3594/359: 0'

780' Cpf g"UT."O qwrkn'U."O kuj tc"U0Kpgtcevkv"dgwy ggp"Q/I nP Ce"o qf kkecvkqp"cpf "v{tqukpg"
r j qur j qt {rvkqp" qh" r tqj kdkkp< lo r rckcvkqp" hqt" c" pqxgn' dlpct { " uy kej 0' RNqU' Qpg0' 422; =
6*4+g67: 80'

790' J cp" GM" O el qpli cn' V." Dwrgt" E." I kcpf c" XN." Nwq" [0' Ej ctcevgtk cvkqp" qh" Cm"
qxgtgzr tguukqp"kp"O kRcEc/4"egm<r tqj kdkkp"ku"cp"Cm"uwdutcvg"dqj "kp"xktq"cpf "kp"egm0'
Cpvkecepgt "Tgu0422: =4: *4C< 79/; 850'

7; 0' Ngg"IG."Mko "M"Uceej gwlpk"IE."Uo kj "EX."Uchg"U0FTR372"eqcevkxcvkqp"qh" gultqi gp" tgegr vqt"cr j c"lp\ T/97"dtgcu'ecpegt"egmu'ku'lpf gr gpf gpv'qh'NZZNN'o qvku0L'Dkqn'Ej go 04227= 4: 2*32+< : 3; /: : 520'

7; 0' Mcvq'O."Vcmckuj k'J ."[qf c'O."Vqj o qpf c'V."Vcnkvq'L'Hwkxc'P."J quqi cpg'P."J qtkvej k'M" Mko wtc"V."Qmfc"["."Uckq"V."Mcy ci vej k'J ."Mkmvej k'V."O cuwo qvq"O."Vq{co c"[."Ej kdc"MO' I TR3" gpj cpegu" gultqi gp" tgegr vqt" cr j c/f gr gpf gpv' gztcegmwxt" o cvkz" i gpg" gzt tguvkqp" kp" ej qpf tqi gple"egm0Quwgqctvj tkku'Ectvkxi g04232=3: *9+< 56/; 630'

820' O wpuvgt"RP."Ectr gpvgt"LV0Gutcf kqn'lp"dtgcu'ecpegt"tgcwo gpv<tgxkxkpi "y g'r cu0LCO CO' 422; =524*9+9; 9/9; : 0'

830' Lqtf cp"XE."Hqtf"NI 0'Rctcf qzkecn'enkplecn'ghge'v'qh" gultqi gp"qp"dtgcu'ecpegt"tkum<c" \$pgy \$"dkmqi { "qh" gultqi gp/kpf wegf "cr qr vuku0Ecpegt "Rt gx'Tgu"*Rj kv -04233=6*7+855/8590'

840' J g"J J ."O g{gt"EC."Ej gp"O Y ."Lqtf cp"XE."Dtqy p"O ."Nkw"Z U0F khtgtpvkcn'F P cug"K j {r gtugpukxkx{ "tgxgcm'hcevt/f gr gpf gpv'ej tqo cvkp"f {pco leu0I gpqo g'Tgu04234=44*8+3237/ 32470'

850' Ctkcl k'GC."Dtckrkq'G."[gttwo "U."Uj wr r "J C."Urkhngt"O L"Ewprkhg"J G."Drcem'OC." Fqpcvq"CN."Ctvgtdwtp"ID."Qr tgc"VK"Rtqupkl'GT."F wp"PL"Lqtf cp"XE0Vj g'I 'r tqvgkp/eqwr ngf" tgegr vqt"I RT52"lpj kdku"r tqrkhtcvkqp"qh" gultqi gp"tgegr vqt/r qukvxg"dtgcu'ecpegt"egm0Ecpegt" Tgu04232=92*5+33: 6/33; 60'

CRRGPF KZ "

30Á[cpi "E\ ."[cpki gt"UK"Lqtf cp"XE."Mrgkp"FL"Dkwpgt"I F0O quv'Rrcukc"Rtqf weu'Tgrgcug" Gultqi gple"Ej go kecn<C"Rqvgpvkn'J gcnj "Rtqdngo "Vj cv'Ecp"Dg"Uqrkf 0'Gpxkqpo gpvcn' J gcnj 'Rgtur gevkgu"4233<33; *9+< : ; /; ; 80'

40ÁLqtf cp"XE."Qdkqtcl "K"Hcp"R."Mko "J T."Dtcwej "J 0'Gxqnwkqp"qh"Nqpi /Vgto "Cf lwxcpv" Cpvkj qto qpg"Vj gter {<Eqpugs wpegu"cpf "Qr r qtwpkkgu<Vj g"U0I cmgp"Rtk g'Ngewtg0' Dtgcw"4233<42*Uwr r n'5+UB/UB30'

50ÁLqtf cp"XE0F gecf gu"qh'F kueqxt {<Vj g"Ugrgevkxg"Gultqi gp"tgegr vqt"O qf wrcvt"*UGTO + "Uqt {<Vj g"U0I cmgp"Rtk g0Tghgtpgu'gp'I {pgeqrqi kg'Qdngt kswg"4233<36<5: 7/; 40'

60ÁJ w\ \ ."Mci cp"DN."Ctkcl k'G."Tqugpy cnF U\ . j cpi "N."Nk'IX."J wpi "J ."Y w'E."Lqtf cp"XE." Tlgi gn'CV."Y gmvgkp"C0'Rtqvgo ke"cpn{uku"qh'r cvj y c{u"lpxqrkf "kp" gultqi gp/kpf wegf " i tqy y "cpf "cr qr vuku"qh'dtgcu'ecpegt"egm0RNqUQPG"4233<Gr wd"8*8+g426320'

70ÁNgy ku/Y co dk'LU."Mko "J ."Ewrcp"T."I tki i "T."Uctngt"OC."Lqtf cp"XE0Vj g'pgy "ugrgevkxg" gultqi gp"tgegr vqt"o qf wrcvt."dc| gf qzkhpg."lpj kdku"j qto qpg/kpf gr gpf gpv'dtgcu'ecpegt" egm' i tqy y " cpf " f qy ptgi wrcvu" gultqi gp" tgegr vqt" " cpf " e{enp" F30' Oqrgewxt" Rjcto ceqrqi { "4233<: 2*6+832/8420'

80ÁI wr c."U0Rtqhkg"qh'X0Etcki "Lqtf cp0Rtqhkg"qh'c'tgegpv{ "grgevgf "o go dgt"qh'y g'P cvkpcn' Cecf go { "qh'Uekpegu"q"cee qo r cp{ "y g'o go dgr'u"kpwi wcn'Ct vkeng0Rt qeggf lpi u'qh'y g" Pcvkpcn'Cecf go { "qh'Uekpegu"WUUC04233<32: *69+3: : 98/3: : 9: 0

90ÁCtkcl k'GC."Ewprkhg"J G."Ngy ku/Y co dk'LU."Urkhngt"O L"Y krku"CN."Tco qu"R."Vcr ke"E." Mko "J T."[gttwo "U."Uj cto c"EI P."Pkeqrnu"G."Drci wtpcvj cp"[."Tquu"GC."Lqtf cp"XE0' Gultqi gp/kpf wegu"Cr qr vuku"kp"Gultqi gp"F gr tkxcvkqp/tgukrcpv'Dtgcu'Ecpegt"xk"Utgau"

- Tgur qpugu" cu" K gpvklgf " d{ " I nqden' I gpg" Gzrtguukqp0' Rtqeggf kpi u" qh' y g" Pcvkqpcn' Cecf go { 'qh'Uekpegu'WUUC04233<32: *69+3: : 9; /3: : : 80
- : 0ÁRqktqv" O 0' Hqwt" f gecf gu" qh" f kueqxt { " kp" dtgcuv" ecpegt" tgugctej " cpf " vgcwo gpv" ó" cp" kpvgtxkgy "y kj "X0'Eteki "Lqtf cp0' kpvgtpcvqpcn'Lqwtpcn'qh'F gxgrqro gpwcn'Dkqrqi { "4233< 77-925/9340
- ; 0ÁQdkqtci "KG."Lqtf cp"XE0Rtqi tguu"kp"gpqf qetkpg"cr r tqcej gu"vq"vj g"vgcwo gpv"cpf "r tngxgvkqp" qh'dtgcuv"ecpegt0O cwt kcu"4233<92-537/5430
- 3200Á czko qx"R[. "Lqtf cp"XE0*4234+"Gutqi gp/Kpf wegf "Cr qr vuku"kp"Dtgcuv"Ecpegt"EgmK" Vtcpuvcvqp"vq"Enkplecn'Tgrxcpeg0Kp<Vcti gvki "P gy "Rcvj y c{u"cpf "Egm'F gcvj "kp"Dtgcuv" Ecpegt" *Tgdgeec'Chv*Gf +0KpVgej . "Tlgnr."Etqcvk."r r "5/440
- 3300Áy ggpg{ "GG."O eF cplgn'TG."O czko qx"R[. "Hcp"R"cpf "Lqtf cp"XE" *4234+"O qf gni"cpf " O gej cpluo u"qh'Ces vktgf "Cpvj qto qpg" Tgukvpcpg"kp"Dtgcuv"Ecpegt<Uki phtkpcv'Enkplecn' Rtqi tguu" F gur kg" Nko kcvkpu0" J qto qpg" O qrgewrt "Dkqrqi { "cpf "Enkplecn'Kpxguiki cvkqp" ; <365/850
- 3400Á cuu"O NU."O cpuqp"LG."Equo cp"H" I tqf uvgkp"H" Lqtf cp"XE."Mtcu" TJ . "Mcvpki "CO . " O cnk"RO . "Uej o kf v'RL"Uj kltgp"LN."Uwgpngn'EC."Wkcp"Y J 0*4234+"Rqukkqp"Ucvgo gpv< Vj g"4234"J qto qpg" Vj gter { "Rqukkqp" Ucvgo gpv" qh" Vj g" P qtvj "Co gtkecp" O gpqr cwug" Uqekgv{ 0O gpqr cwug<Vj g'Lqwtpcn'qhVj g'P qtvj "Co gtkecp" O gpqr cwug"Uqekgv{ 3; <479/930'
- 3500Á g"J J . "O g{gt"EC."Ej gp"O Y . "Lqtf cp"XE."Dtqy p"O . "Nkw"Z U0'F khtgtpvkn'F P cug"K j { r gtugpukxkx{ "tgxgcn" hcvqt/f gr gpf gpv'ej tqo cvk" f { pco leu0' I gpqo g" Tgugctej "4234" *Grwd"cj gcf "qlhrtkpv0'
- 3600Á Lqtf cp."XE0*4234+"Gutqi gp"Cevkqp."UGTO u"cpf "Y omen'u"J gcnj 0'Y qtrf "Uekpvkhe0' P gy "Lgtug{ . "Nqpf qp."Ukpi cr qtg" *kp"rtgrctcvkqp+0'
- 3700Á Lqtf cp."XE"cpf "O czko qx"R[" *4234+"Vco qz kpg/Rkqpggtkpi "O gf kelpg"kp"Dtgcuv"Ecpegt0' O kguvqpgu"kp" F twi "Vj gter { 0'Ugtkgu" Gf u<Rctpj co "O L"cpf "Dtwkpxgnu"l0'Ur tkpi gt" Dcugn' CI . "Dcuen"Uy k{ gtrcpf " *kp"rtgrctcvkqp+0"
- 3800Áy ggpg{ "G"cpf "Lqtf cp"XE0*4234+"Gutqi gp" Tgegr vqt " *GT +0Kp<Gpe{ emr gf kc"qh'Ecpegt" Vj gter gwke"Vcti gu0'Ur tkpi gt"Uekpegt"- "Dwukpguu"O gf kc."NNE."P gy "l qtm" *kp"rtguu+0'
- 3900Á eF cplgn'T . "Lqtf cp"XE0*4234+"Gpf qetkpg"Rtngxgvkqp"qh"Dtgcuv"Ecpegt0Kp<Gctn{ "Dtgcuv" Ecpegt<Htqo "Uetggplki "vq"O wnkf kuekr nqct { "O cpci go gpv0'Kphqto c"J gcnj ectg."Nqpf qp." Gpi ncpf " *kp"rtguu+0'
- 3: 0Ácp"R."O eF cplgn'TG."Mko "J T."Emi gw"F."J cf f cf "D."Lqtf cp"XE0*4234+"O qf wrcvki " Vj gter gwke" Gthgevu"qh" y g"e/Ute" Kp j kdkqt" xlc" Gutqi gp" Tgegr vqt"cpf "J GT4"kp"Dtgcuv" Ecpegt"Egm'Nkpgu0'Gwtqr gcp" Lqwtpcn'qh'Ecpegt" *kp"rtguu+0'
- 3; 0Ádrci wtwpvj cp"[. "O qwuugu"U."Dkvpgt"O N0*422: +ukTP C"Uetggplki <C"Rtqeguu"O qf gni' vq" Gxcnvcg"J k" Tcvg" F kueqxt { 0'Rtguqvgf "cpf "r wdrukj gf "kp"cuqekcvkqp"y kj "vj g"422: " KGG'I gpqo leu"Uki pcn'Rtqeguulpi "cpf "Ucvkukleu" *I GP UkRU+Kpvgtpcvqpcn'Y qtmij qr 0'

"

Most Plastic Products Release Estrogenic Chemicals: A Potential Health Problem that Can Be Solved

Chun Z. Yang,¹ Stuart I. Yaniger,² V. Craig Jordan,³ Daniel J. Klein,² and George D. Bittner^{1,2,4}

¹CertiChem Inc., Austin, Texas, USA; ²PlastiPure Inc., Austin, Texas, USA; ³Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA; ⁴Neurobiology Section, School of Biology, University of Texas, Austin, Texas, USA

BACKGROUND: Chemicals having estrogenic activity (EA) reportedly cause many adverse health effects, especially at low (picomolar to nanomolar) doses in fetal and juvenile mammals.

OBJECTIVES: We sought to determine whether commercially available plastic resins and products, including baby bottles and other products advertised as bisphenol A (BPA) free, release chemicals having EA.

METHODS: We used a roboticized MCF-7 cell proliferation assay, which is very sensitive, accurate, and repeatable, to quantify the EA of chemicals leached into saline or ethanol extracts of many types of commercially available plastic materials, some exposed to common-use stresses (microwaving, ultraviolet radiation, and/or autoclaving).

RESULTS: Almost all commercially available plastic products we sampled—independent of the type of resin, product, or retail source—leached chemicals having reliably detectable EA, including those advertised as BPA free. In some cases, BPA-free products released chemicals having more EA than did BPA-containing products.

CONCLUSIONS: Many plastic products are mischaracterized as being EA free if extracted with only one solvent and not exposed to common-use stresses. However, we can identify existing compounds, or have developed, monomers, additives, or processing agents that have no detectable EA and have similar costs. Hence, our data suggest that EA-free plastic products exposed to common-use stresses and extracted by saline and ethanol solvents could be cost-effectively made on a commercial scale and thereby eliminate a potential health risk posed by most currently available plastic products that leach chemicals having EA into food products.

KEY WORDS: bisphenol A, endocrine disruptor, endocrine-disrupting chemical, estrogen receptor binding, estrogenic activity, plastic. *Environ Health Perspect* 119:989–996 (2011). doi:10.1289/ehp.1003220 [Online 2 March 2011]

Chemicals that mimic or antagonize the actions of naturally occurring estrogens are defined as having estrogenic activity (EA), which is the most common form of endocrine disruptor activity [Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) 2003, 2006; National Research Council 1999]. Chemicals having EA typically interact with one or more of the classical nuclear estrogen receptor (ER) subtypes: ER α , ER β , or nonclassical membrane or ER-related subtypes (Hewitt et al. 2005; Matsushima et al. 2008; National Research Council 1999). In mammals, chemicals having EA can produce many health-related problems, such as early puberty in females, reduced sperm counts, altered functions of reproductive organs, obesity, altered sex-specific behaviors, and increased rates of some breast, ovarian, testicular, and prostate cancers (Della Seta et al. 2006; Gray 2008; Kabuto et al. 2004; National Research Council 1999; Newbold et al. 2004; Patisaul et al. 2006, 2009). Fetal, newborn, and juvenile mammals are especially sensitive to very low (sometimes picomolar to nanomolar) doses of chemicals having EA (Gray 2008; vom Saal et al. 2005). Many of these effects observed in mammals are also expected to be produced in humans, because basic endocrine mechanisms have been highly conserved across all classes

of vertebrates (Kavlock et al. 1996; National Research Council 1999).

Thermoplastics, which are used for many items that contain food, are made by polymerizing a specific monomer or monomers in the presence of catalysts into a high-molecular-weight chain known as a thermoplastic polymer [see Supplemental Material, Figure 1 (doi:10.1289/ehp.1003220)]. The resulting polymer is mixed with small quantities of various additives (antioxidants, plasticizers, clarifiers, etc.) and melted, mixed, extruded, and pelletized to form a base thermoplastic resin. Base resins are either used as is [e.g., bisphenol A (BPA)-based polycarbonate (PC), non-BPA-based polypropylene (PP) copolymer (PPCO), and non-BPA-based PP homopolymer (PPhO)] or, more commonly, mixed with other resins, additives, colorants, and/or extenders to form plastic compounds (e.g., polymer blends and precolored polymers). Plastic products are then made by using one or more plastic compounds or resins to form a finished plastic part that can be subjected to finishing processes that may use inks, adhesives, and so forth, to make a finished product.

As previously described (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004), plastic resins and manufacturing protocols [see Supplemental Material, Figure 1 (doi:10.1289/ehp.1003220)] collectively use many

monomers and additives that may exhibit EA because they have physicochemical properties, often from an insufficiently hindered phenol (HP) group, that enable them to bind to ERs (see Supplemental Material, Table 1). Because polymerization of monomers is rarely complete and additives are not chemically part of the polymeric structure, chemicals having EA can leach from plastic products at very low (e.g., nanomolar to picomolar) concentrations that individually or in combination can produce adverse effects, especially in fetal to juvenile mammals. This leaching of monomers and additives from a plastic item into its contents is often accelerated if the product is exposed to common-use stresses such as ultraviolet (UV) radiation in sunlight, microwave radiation, and/or moist heat via boiling or dishwashing. The exact chemical composition of almost any commercially available plastic part is proprietary and not known. A single part may consist of 5–30 chemicals, and a plastic item containing many parts (e.g., a baby bottle) may consist of ≥ 100 chemicals, almost all of which can leach from the product, especially when stressed. Unless the selection of chemicals is carefully controlled, some of those chemicals will almost certainly have EA, and even when using all materials that initially test EA free, the stresses of manufacturing can change chemical structures or create chemical reactions to convert an EA-free chemical into one with EA.

Address correspondence to G.D. Bittner, CertiChem, Inc., 11212 Metric Blvd., Suite 500, Austin, TX 78758 USA. Telephone: (512) 339-0550, ext. 201; Fax: (512) 339-0551. E-mail: gbittner@certichem.com

Supplemental Material is available online (doi:10.1289/ehp.1003220 via <http://dx.doi.org/>).

This work was supported by National Institutes of Health (NIH) grants R44 ES011469 (01-03) and 1R43/44 ES014806 (01-03) to C.Z.Y.; R44 ES016964 (01-03) to S.I.Y.; and P30 CA051008 to V.C.J.

C.Z.Y. is employed by, and owns stock in, CertiChem (CCi) and PlastiPure (PPi). S.I.Y. and D.J.K. are employed by PPi. V.C.J. has no financial interests in CCi or PPi, but he was principal investigator for a subcontract at Northwestern Medical School to help develop the MCF-7 assay on NIH grant P30 CA051008 awarded to CCi. G.D.B. owns stock in, and is the founder and chief executive officer of CCi and the founder and chief scientific officer of PPi. All authors had freedom to design, conduct, interpret, and publish research uncompromised by any controlling sponsor.

Received 16 November 2010; accepted 24 February 2011.

Very few studies (Soto et al. 1991; Till et al. 1982) have examined the extent to which plastics that presumably do not contain BPA nevertheless release other chemicals having detectable EA. For example, a recent comprehensive review [table on page 72 of Gray (2008)] described polyethylene (PE), PP, and PE terephthalate (PET) plastics as being “OK” for use with respect to release of chemicals exhibiting EA.”

Here, we report that most of the > 500 commercially available plastic products that we sampled—even those that are presumably BPA free—release chemicals having detectable EA, especially if they are assayed by more polar and less polar solvents and exposed to common-use stresses. That is, we show that, to reliably detect such leachable chemicals having EA, unstressed or stressed plastic resins or products should be extracted with more polar (e.g., saline) and less polar [e.g., ethanol (EtOH)] solutions and exposed to common-use stresses (boiling water, microwaving, and UV radiation).

Materials and Methods

We developed a sensitive and accurate robotized version of the MCF-7 cell proliferation assay (E-SCREEN assay) that has been used for decades to reliably assess EA and anti-EA

(Leusch et al. 2010; Soto et al. 1995) and is currently undergoing validation for international use by ICCVAM/NTP (National Toxicology Program) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). Chemicals with EA bind to ERs (ER α , ER β , or ER-related subtypes) and activate the transcription of estrogen-responsive genes, which leads to proliferation of MCF-7 cells.

Detailed methods for the MCF-7 assay are provided in Supplemental Material, (doi:10.1289/ehp.1003220). In brief, plastic resins or products were extracted using saline, a more polar solvent, or EtOH, a less polar solvent. Aliquots of the extracts were then diluted four to eight times to produce up to eight test concentrations. Each test chemical or extract at each concentration was added in triplicate or quadruplicate to 96-well plates containing MCF-7 cells in EA-free culture media. After 6 days of exposure, the amount of DNA per well, an indication of cell proliferation, was assayed using a microplate modification of the Burton diphenylamine assay (Burton 1956; Natarajan 1994).

The effect of a test chemical or extract on proliferation was expressed as the %E2, a percentage of the maximum DNA per

well produced by the maximum response to 17 β -estradiol (E2; positive control) corrected by the DNA response to the vehicle (negative) control [see Supplemental Material, Equation 1 (doi:10.1289/ehp.1003220)]. For estrogenic test chemicals, the concentration needed to obtain half-maximum stimulation of cell proliferation [half-maximal effective concentration (EC₅₀), a measure of binding affinity] was calculated from best fits to dose–response data that meet a well-defined set of criteria by Michaelis-Menton kinetics. The estrogenicity of extracts was calculated as the relative maximum %E2 (%RME2; a measure of response amplitude), a percentage of the maximum DNA per well produced by an extract at any dilution with respect to the maximum DNA per well produced by E2 at any dilution, corrected by the DNA response to the vehicle (negative) control (see Supplemental Material, Equation 2). If a test chemical had a positive response (> 15% RME2) but an EC₅₀ could be calculated because not all criteria were met, then the estrogenicity of the test chemical was characterized simply as EA positive or by its %RME2.

The EA of a test chemical or extract was considered detectable if it produced cell proliferation > 15% of the maximum response to E2 (> 15% RME2), which is > 3SDs

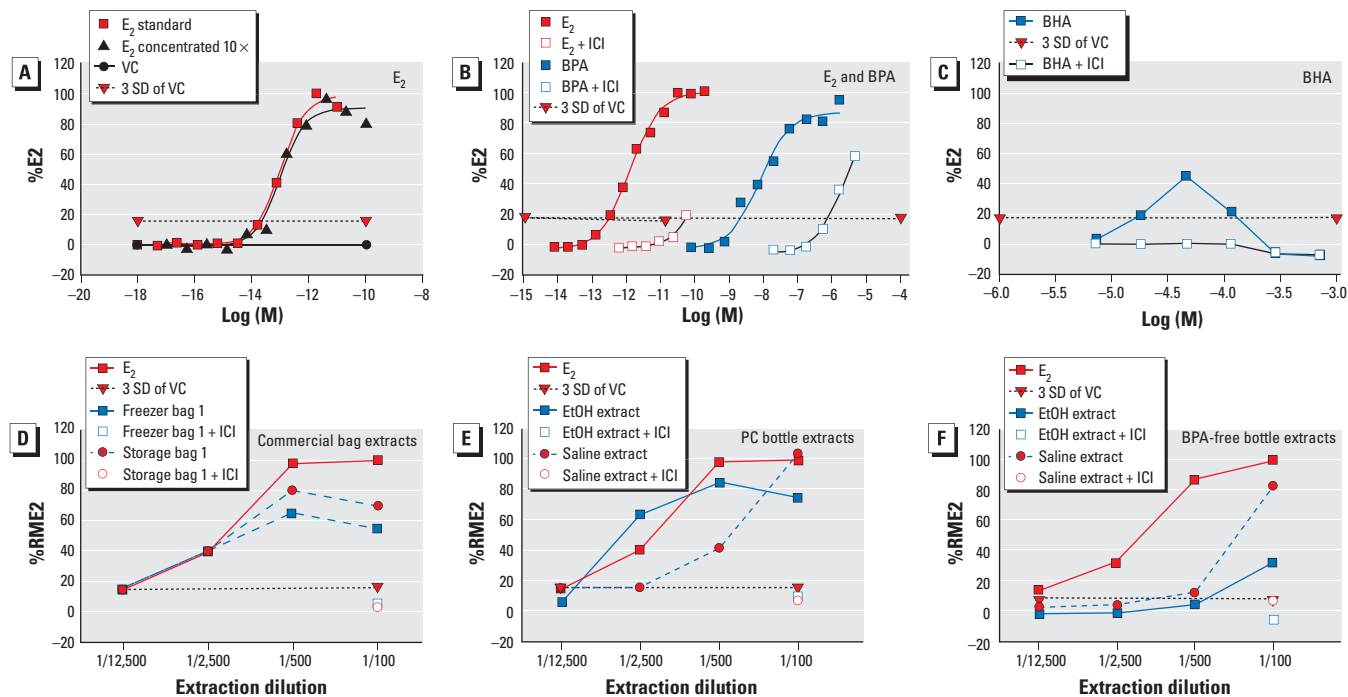


Figure 1. Results of MCF-7 assays shown as dilution response curves (%E2) for E2 (A), E2 and BPA (B), BHA (C), and %RME2 of extracts of plastic bags (D), a PC bottle (E), and a BPA-free bottle made from PETG (F). Abbreviations: PETG, PET glycol-modified polyethylene terephthalate; VC, vehicle control. Dotted lines represent 3 SD from the response. In B–F, the negative control (1% EtOH or saline) equals 0% E2. The E2 standard (10^{-9} M) is the positive control diluted as indicated in C–F. Each point plotted is the average of three or four replicates for each concentration whose SD is very small and falls within the space taken up by each data point. In (A), E2 was dissolved in EtOH (standard extract) or concentrated 10 \times and rediluted to show that the EtOH concentration protocol has very little effect on the EC₅₀ of E2 (50% E2). The EC₅₀ of E2 is approximately 1.3×10^{-13} M, and the threshold of detection (15% E2) is approximately 10^{-15} M. The maximum E2 response was attained at 10^{-11} M and remained constant at higher E2 concentrations. (B) The EC₅₀ of both E2 (as in A) and BPA is approximately 6.6×10^{-8} M, and threshold detection is approximately 10^{-9} M, all suppressed by 10^{-8} M ICI. (C) BHA does not meet criteria needed for accurate calculation of EC₅₀ [see Supplemental Material, pp. 5–7 (doi:10.1289/ehp.1003220)]. EA is positive; its maximum response is about 50% E2 (i.e., 50% RME2) and is suppressed by 10^{-8} M ICI. In D, commercially available plastic bags were extracted by 100% EtOH. Commercially available PC (E) and BPA-free (F) bottles were extracted with saline or EtOH as indicated.

from the historic control baseline response (about 10^{-15} M), which is a rather conservative measure of EA detectability. Stimulation of MCF-7 proliferation induced by the test chemical or extract was confirmed to be estrogenic (compared with nonspecific) in an EA confirmation study: If the stimulation of MCF-7 proliferation by a test chemical or extract was suppressed by coincubation with a strong antiestrogen [ICI 182,780 (ICI) at 10^{-7} to 10^{-8} M], the EA of the test chemical or extract was confirmed. Therefore, a test chemical or extract was classified as not having detectable EA if it did not induce MCF-7 cell proliferation or if it induced proliferation that could not be inhibited by ICI.

Figure 1 shows typical MCF-7 responses plotted as %E2. Figure 1A–C show responses to some test chemicals: E2 (positive control), BPA, and butylated hydroxyanisole (BHA; a common antioxidant). Figure 1D–F show %RME2 responses to test extracts of plastic food bags, PC bottles, and BPA-free baby bottles and their ICI-suppressed responses, confirming their EA. Some chemicals or products were also analyzed for anti-EA [for details, see Supplemental Material, pp. 7–8 (doi:10.1289/ehp.1003220)].

Purchase and analyses of plastic products in survey studies. For Tables 1 and 2, we purchased 455 plastic products used to contain foodstuffs from various commercial retailers from 2005 through 2008. The relative frequency of products having detectable EA did not change with later compared with earlier purchases. In some cases, we instructed undergraduate students or employees to purchase a mix of plastic items used to contain foodstuffs from a given large retailer (Albertsons, H-E-B, Randalls, Target, Wal-Mart, Trader Joe's, and Whole Foods) mainly in the Austin, Texas, or Boston, Massachusetts, areas, some of which market many "organic" products. In other cases, we purchased products of a particular plastic type (e.g., PE- or PP-based containers). We recorded the retailer, resin type [high-density PE (HDPE), PET, PC, PP, polystyrene (PS), polylactic acid], and product type (flexible packaging, food wrap, rigid packaging, baby bottle component, deli containers, plastic bags). In addition, because the contents of some plastic items might have added or extracted chemicals having EA from the plastic containers before we purchased and tested the products (Sax 2010), we recorded whether the plastic items had contents or were empty when purchased. For any plastic container having contents, we thoroughly washed out the container with distilled water before testing the plastic. Except for PC-based items, none of these products were known to contain BPA. (Plastic products typically do not list their chemical composition, which is proprietary to the manufacturer.) Samples were chosen in product areas where adverse health effects

might occur if the samples leached chemicals having EA. Samples from each retailer generally included most of the product types listed above. In addition to surveying commercially available products, we tested plastic resins [e.g., PC, PET, glycol-modified PET (PETG)] that were purchased from M. Holland Company (Northbrook, IL) and individual chemicals used to manufacture plastic products [e.g., BPA, BHA, butylated hydroxytoluene (BHT), dimethyl terephthalate, etc.] that were purchased in their purest form from Sigma-Aldrich (St. Louis, MO).

Many plastic products have more than one plastic part. For example, baby bottles have 3–10 different plastic parts in various combinations [bottle, nipple, anticolic item(s), sealing ring(s), liner bag, cap, etc.], each part typically having different and rather unique combinations of 5–30 chemicals. Over the course of this entire study, we assayed > 100 component parts from > 20 different baby bottles, including many advertised as BPA free. Only some (13) of these component parts were purchased for the initial survey study (Tables 1 and 2).

Table 1. Percentage of unstressed plastic products having EA in at least one extract.

Plastic product	Extraction solvent							
	EtOH		Concentrated EtOH		Saline		Any extract	
	<i>n</i>	%D	<i>n</i>	%D	<i>n</i>	%D	<i>n</i>	%D
Resin type								
HDPE	13	69	11	55	18	56	30	70
PP	23	52	6	33	16	81	37	68
PET	30	40	17	94	34	76	57	75
PS	13	62	—	—	16	38	28	50
PLA	10	70	1	100	8	100	11	91
PC	1	0	1	100	2	100	2	100
Product type								
Flexible packaging	82	66	6	33	35	74	121	67
Food wrap	9	100	—	—	9	78	9	100
Rigid packaging	57	56	18	67	31	45	83	64
Baby bottle component	13	69	—	—	16	94	19	89
Deli containers	11	36	—	—	7	7	16	44
Plastic bags	33	97	1	100	23	96	43	98
Product retailer								
Large retailer 1	31	81	2	100	4	75	36	81
Large retailer 2	4	50	4	0	50	54	53	53
Large retailer 3	18	83	2	100	7	29	25	72
Large retailer 4	37	51	—	—	—	—	37	51
Large retailer 5	20	50	3	100	4	100	23	70
Organic retailer 1	28	71	5	60	5	80	32	81
Organic retailer 2	33	88	1	100	10	80	35	89
Total for extract	308	68	51	73	214	69	455	72

Abbreviations: —, not tested; %D, percent detectable (extract produced cell proliferation > 15% RME2; see "Materials and Methods"); *n*, total number of samples purchased (less than the sum of *n* values for individual extracts if some items were tested by more than one extraction protocol); PLA, polylactic acid. Data are percentages of samples for which EA was detected using a standard or concentrated EtOH extract, a saline extract, or one or more such extracts (any extract). Some individual items are listed in two or three categories (e.g., PET and baby bottles) but were counted only once for the extract total. Baby bottle components comprised 11 bottles and 2 sealant ring components.

Table 2. Percentage of unstressed plastic products having detectable EA (> 15% RME2) in two extracts.

Category	<i>n</i>	Extraction solvent			
		EtOH only	Saline only	Both EtOH and saline	Either EtOH or saline
HDPE	13	15	31	15	61
PET	21	19	29	52	100
PP	4	0	25	75	100
PLA	7	0	14	86	100
Bottles	38	13	34	42	89
Baby bottles	11	0	36	64	100
Rigid packaging	10	30	20	40	90
Food wrap	8	25	0	75	100
All products	102	17	21	54	92

PLA, polylactic acid. Values shown are percent (%) of unstressed plastic items (*n*) having detectable EA (> 15% RME2) only in an EtOH extract (and not in a saline extract), only in a standard saline extract (and not in an EtOH extract), in both EtOH and saline extracts, or in either EtOH or saline extracts. The last column is the sum of the three previous columns. "All products" is the total for each column when each product (*n* = 102) is only counted once (some products are listed in two categories). The standard EtOH extract was used for most (*n* = 81) products and the concentrated EtOH extract for the remainder (*n* = 21). If EA was detected in a saline or standard EtOH extract in survey studies such as those reported in Table 1, other extracts often were not performed. A concentrated EtOH extract was usually used to generate data shown in Tables 1 and 2 only if EA was not detected in a saline or standard EtOH extract. That is, samples listed for concentrated EtOH in Table 1 and EtOH in Table 2 had a selection bias for not having detectable EA.

Most of the samples (338 of 455) in the survey study (Tables 1 and 2) were extracted using only one extraction protocol. For the remaining samples ($n = 102$), both saline and EtOH extractions were used so that the efficacy of each protocol could be directly compared. We used a paired Student's t -test to test whether differences between pairs of samples were statistically significant ($p < 0.05$).

Protocols for common-use stresses of some plastic items. Given that common-use stresses can alter the complex chemical composition of plastics and/or increase the rate of leaching (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004), for some resins or products, we examined how leaching of chemicals having EA might be affected by exposure to microwave radiation, autoclaving (moist heat), and UV light. Additional plastic items, some of which are described in Figure 2 and Table 3, were purchased in 2008–2010 and subjected to common-use stresses. In addition, we tested a variety of resins (including PE- and PP-based resins; Table 3), antioxidants [see Supplemental Material, Table 3 (doi:10.1289/ehp.1003220)],

and other additives or processing agents (see Supplemental Material, Table 4) identified by our laboratory as being free of detectable EA and hence possibly suitable for use to produce final products that would be EA free even after exposure to common-use stresses.

We used the following stresses:

- Samples were placed about 2 feet from a 254-nm fluorescent fixture for 24 hr, simulating repeated UV stress by sunlight (e.g., water bottles) or UV sterilizers (e.g., baby bottles and medical items)
- Samples were autoclaved at 134°C for 8 min, simulating moist heat stress in an automatic dishwasher
- We heated samples in a microwave 10 times for 2 min each, using a 1,000-W kitchen microwave oven set to “high,” simulating heat and microwave radiation stress to reusable food containers.

Results

Release of chemicals having EA from unstressed plastics. Tables 1 and 2 show the percentage of samples in each category that had reliably

detectable EA ($> 15\%$ RME2) in our survey of 455 commercially available plastic products. [For the %RME2 and content status of individual samples, as well as the average %RME2 for products classified by resins (HDPE, PP, PET, PS, polylactic acid, PC), product type (flexible packaging, food wrap, rigid packaging, baby bottle components, plastic bags), and retailer (large retailers 1–5 and large organic retailers 1 and 2), see Supplemental Material, Table 5 (doi:10.1289/ehp.1003220).] For example, 9 of 13 HDPE plastic products extracted by our standard EtOH protocol (69%) had detectable EA (Table 1), with a %RME2 (mean \pm SD) of $66\% \pm 25\%$ (see Supplemental Material, Table 5A). For PET products extracted by saline, 26 of 34 (76%) had detectable EA (Table 1) with a %RME2 of $64\% \pm 41\%$ (see Supplemental Material, Table 5C). We found no consistent correlation between the percentage of items in a product type with detectable EA and their mean %RME2 (data not shown).

We found no significant difference ($p > 0.05$) in the percentage of items with detectable EA between those with contents and those with no contents (76%, $n = 160$) at the time of purchase based on the standard EtOH extraction protocol [67% vs. 70%; see Supplemental Material, Table 2A (doi:10.1289/ehp.1003220)], the standard saline protocol (62% vs. 75%; see Supplemental Material, Table 2C), or all extraction protocols combined (69% vs. 76%). Most important, items with no contents in all categories exhibited detectable EA in at least one protocol (see Supplemental Material, Tables 2 and 5), including 78% of items made from HDPE ($n = 18$), 57% from PP ($n = 14$), and 100% from PET ($n = 6$). Given all of these results, we present the data for all items shown in Tables 1 and 2 without regard to their content status.

Using different solvents increased the probability of detecting EA. Most (71%) unstressed plastic items released chemicals with reliably detectable EA in one or more extraction protocols, independent of resin type, product type, or retailer (Table 1). Results often differed between saline and EtOH extracts of the same unstressed plastic item, and EA was reliably detected most frequently (92% of all items listed in Table 2) when analyzed using both saline (more polar) and EtOH (less polar) extracts. For example, 15% of unstressed HDPE plastic items leached chemicals with detectable EA into both EtOH and saline extracts, 15% leached only into EtOH, and 31% leached only into saline (Table 2). That is, the leaching of a chemical with EA was significantly ($p < 0.01$) more likely to be detected if we used both polar and non-polar solvents (61%) than if we used only one solvent (30% for EtOH only or 45% for saline

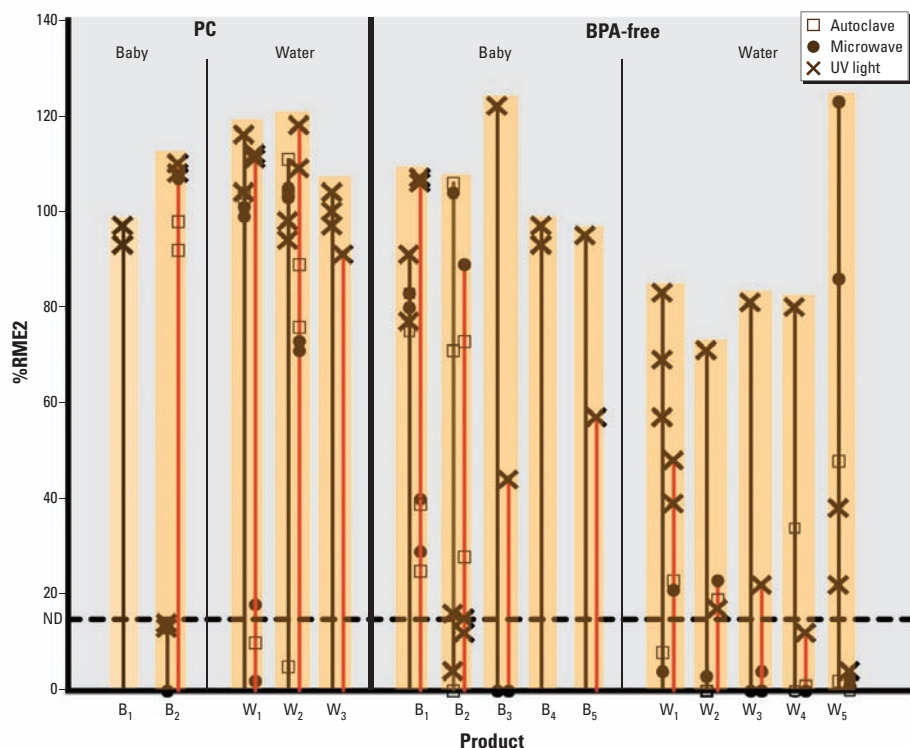


Figure 2. Total EA released by some PC and BPA-free water bottles (W) and baby bottles (B). The leaching of chemicals having EA (measured as %RME2; excluding caps, nipples, and other components) were extracted using saline or EtOH as solvents and exposed to autoclaving, microwaving, and/or UV light (see “Materials and Methods” for details). BPA-free water bottles W₁, W₂, W₃, and W₄ are PETG, and W₅ is PET. BPA-free baby bottles B₁ and B₂ are polyethersulfone; B₃ is PETG; and B₄ and B₅ are PP. Orange bars indicate the data set for each individual product. The %RME2 for saline extracts is represented by solid black lines and for EtOH as solid red lines. Symbols represent the %RME2 of chemicals released by each assay of a product after an autoclaving stress, microwaving stress, and UV light stress (see figure key). The dotted horizontal line at 15% RME2 is the rather conservative value below which EA was considered nondetectable (ND) for any assay. For some products shown (e.g., PC B₁, BPA-free B₄), if one solvent and/or stress condition showed reliably detectable EA, other solvents and stress conditions were not subsequently tested. Some values plotted as 0% RME2 actually had slightly negative %RME2 values (–1% to –7% RME2) due to cellular toxicity.

only). We obtained similar results for all types of plastic products (data not shown).

Assays of > 100 component parts from > 20 different baby bottles, including many advertised as BPA free, indicated that extracts of at least one bottle component of each baby bottle always had EA based on at least one assay (some data shown in Table 2 and Figure 2), as did at least one other component part (data not shown).

Stresses increased the release of chemicals having EA. Leaching of chemicals with EA was increased by common stresses. For example, one unstressed sample of an HDPE resin (P5 in Table 3) that had no detectable EA (i.e., RME2 < 15%) in two saline extracts and two EtOH extracts released chemicals with EA equivalent to 47% RME2 when extracted using EtOH after the resin was stressed with UV light. Similarly, two samples of low-density PE resins (LDPE resins 1 and 2) and PETG resins (PETG baby bottle and PETG resin 1) that had no detectable EA before stressing subsequently exhibited EA when stressed, especially by UV (Table 3). Samples ($n > 10$) of products made from PETG resins advertised as BPA free all released detectable EA when stressed, especially by UV light. Similarly, 25% of unstressed samples of PET and 50% of unstressed PS products surveyed did not have detectable EA in assays of EtOH and/or saline extracts (Table 1). However, when stressed and assayed using both saline and EtOH extracts, all PET ($n > 10$) and PS ($n > 10$) products released chemicals having detectable EA in at least one extracting solvent (Table 3).

EA-containing and EA-free monomers. Polymerization of monomers is rarely complete, and unpolymerized monomers are almost always released from polymer resins (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004). PE and PP polymers are often used to manufacture flexible and/or nontransparent rigid products (Figure 3). MCF-7 assays ($n = 6$) consistently showed that extracts of "barefoot" (no additives) polymers (e.g., LDPE resin P1 in Table 3) were EA free, even when stressed. (PP-based polymers require antioxidants to prevent severe degradation during their use in manufacturing plastic products.) Furthermore, PE- and PP-based resins containing appropriate additives to produce fit-for-use products could be constructed that remained EA free ($n > 100$ assays of > 10 resins), even when exposed to common-use stresses. Representative data from several such resins (LDPE resin P1, HDPE resin P2, PP homopolymer resin P3, PP copolymer resin P4) are shown in Table 3.

Figure 3 also shows other monomers and polymers that can or cannot be used to make hard-and-clear (HC) plastics. For example, HC PC plastics ($n > 10$) all released chemicals having EA (e.g., PC baby bottle B₁ and

PC water bottle W₁ in Figure 2), almost certainly phenolics such as BPA (Figure 1B). The dimethyl terephthalate monomer used to make PET and PETG plastics exhibited anti-EA ($n = 3$ assays; data not shown; for anti-EA assay protocol, see Supplemental Material (doi:10.1289/ehp.1003220)). Furthermore, breakdown products of dimethyl terephthalate, PET, and PETG resins probably contain and release phenolic moieties that have EA that account for some of the data for PET products in Tables 1 and 2. Polyethersulfone HC products also consistently released chemicals having EA or anti-EA, especially when stressed with UV light (data not shown), possibly from unreacted phenolic monomer residues or phenolic stress-degradation products. In contrast, some HC cyclic olefin polymer/cyclic olefin copolymer polymers produced from saturated cyclic olefin monomers contained no phenolics and did not release chemicals having detectable EA, even when stressed (Table 3).

Polymers that can be made EA free have a similar cost compared with polymers made from monomers that have EA. For example, currently, clarified PP having no additives that exhibit EA (even when stressed) that is suitable for molding bottles costs approximately \$1.20/lb. PP resins containing additives that have EA also cost about \$1.20/lb. Commodity resins such as PET, which are made from monomers having EA and are suitable for molding bottles, are priced at approximately \$1.28/lb (Plastics News 2011).

EA-containing and EA-free additives. Many additives are physically, but not

chemically, bound to a polymeric structure and hence can almost always leach from the polymer, especially when stressed (Begley et al. 1990, 2005; De Meulenaer and Huyghebaert 2004). Antioxidants are the most critical class of additives because they prevent or minimize plastic degradation due to oxidation that breaks polymer chains (chain scission) and/or causes cross-links (Kattas et al. 2000). The oldest and most common antioxidants deemed suitable for food contact belong to a chemical class known as HPs (hindered phenols), such as BHT and BHA, in large part because both are inexpensive and assumed to be nontoxic. However, BHT ($n = 4$ assays) had reliably detectable EA, as did BHA ($n = 3$ assays). [The EC₅₀ of BHT and BHA (Figure 1C) could not be accurately calculated because both also exhibited cellular toxicity at higher concentrations (10⁻⁵ M).] Other commonly used HP antioxidants ($n = 4/5$) and organophosphines ($n = 6/7$) also exhibited reliably detectable EA, especially when exposed to moist heat, which presumably causes hydrolysis (data not shown). For example, proprietary antioxidants Phos (phosphate) OX 1 and HP AOX 2 had no detectable EA, whereas HP AOX 1 and Ph (bisphenol) AOX 1 had reliably detectable EA [see Supplemental Material, Table 3 (doi:10.1289/ehp.1003220)].

Many other additives ($n > 50$) with a phenolic group had reliably detectable EA, such as agents found in many base resins [tris(nonylphenyl) phosphite, octylphenol, nonylphenol, butylbenzene phthalate], colorants (especially blues or greens with

Table 3. Representative %RME2 values for stressed resins or parts made from flexible or HC polymers.

Sample type	Stress/extraction solvent					
	Microwave		UV		Autoclave	
	Saline	EtOH	Saline	EtOH	Saline	EtOH
Flexible polymers						
LDPE resin 1	5	7	0	4	4	30 ^a
LDPE resin 2	3	7	26 ^a	3	-1	27 ^a
PET water bottle	100 ^a	3	31 ^a	2	47 ^a	1
LDPE resin P1	2	3	0	0	4	5
HDPE resin P2	6	-4	2	-2	-1	-3
PPHO resin P3	0	-4	3	2	-6	-3
PPCO resin P4	3	7	-7	-6	-9	-3
HDPE resin P5	ND	ND	ND	47 ^a	ND	ND
HC polymers						
Water bottle 1.1	3	23 ^a	71 ^a	17 ^a	-1	19 ^a
Water bottle 1.2	4	21 ^a	57, ^a 69, ^a 98 ^a	48, ^a 39 ^a	8	23 ^a
Water bottle 2.1	-7	-5	81 ^a	22 ^a	0	4
Water bottle 2.2	34 ^a	-2	80 ^a	12	-1	1
PETG baby bottle	0	-2	122 ^a	44 ^a	0	1
PETG resin 1	-8	17 ^a	61 ^a	111 ^a	0	15 ^a
PS 1	4	3	17 ^a	45 ^a	76 ^a	0
COC 3	9	7	20 ^a	20 ^a	0	6
COC resin P18	4	1	9	11	1	-2
COC resin P19	6	2	6	-2	4	2

Abbreviations: COC, cyclic olefin copolymer; ND, not determined; PPCO, polypropylene copolymer; PPHO, polypropylene homopolymer. Numerical values are %RME2 responses of extract for several different baby bottle and other component parts. Resins designated with P (e.g., P1, P18) are EA-free formulations developed at PlastiPure. Resin P5 exhibited EA when stressed. Multiple values for water bottle 1.2 under UV stress are data for repeated analyses.

^aPlastic items leaching chemicals having detectable EA > 15% RME2.

phthalocyanine groups), PS-based purge compounds, and mold-release agents [see Supplemental Material, Table 4 (doi:10.1289/ehp.1003220)]. In contrast, many metal-oxide-based inorganic pigments did not exhibit EA. However, these EA-free pigments are often mixed with dispersing agents and carrier resins that have EA to produce colorant masterbatch concentrates. Nevertheless, we have identified resins, dispersants, pigments, and antioxidants that are approved by the Food and Drug Administration for direct food contact (see Supplemental Material, Tables 3 and 4) to create colorant masterbatch concentrates ($n > 100$) that produce

even colorant dispersion into plastics and that have no detectable EA, cellular toxicity, or adverse processing effects, even when stressed.



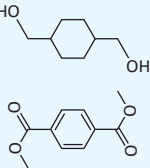
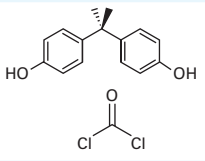
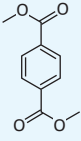

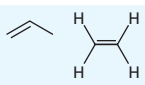
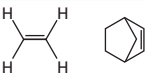

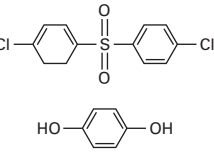
Because additives comprise a small fraction (typically 0.1–1% by weight) of plastic resins and compounds and because plastic resins and compounds using EA-free additives are processed during manufacture in a nearly identical manner as conventional resins and compounds containing chemicals with EA, the replacement of additives having EA with EA-free additives should have very little impact on the cost of the final product. Furthermore, EA-free additives have only a slightly higher or no additional cost compared

with additives with EA, so that their cost impact is very small or nonexistent.

Products currently marketed as BPA free are not EA free. In response to market and regulatory pressures to eliminate BPA in HC plastics, BPA-free HC materials have recently been introduced as replacements for PC resins. PET and PETG are two such resins, but HC plastic products made from these resins leached chemicals that had detectable EA (Tables 1–3, Figures 2 and 3), often in the absence of exposure to common-use stresses. Two popular brands of water bottles made from a PETG resin now marketed as an HC BPA-free replacement also released chemicals having significant EA (W1, W2, W3, and W4; Table 3, Figures 2 and 3), as did uncompounded PETG resins (Table 3). Most PE/PP-based plastic products were presumably BPA free but nevertheless had readily detectable EA (Tables 1 and 2), almost certainly due to one or more additives having EA. Many components of BPA-free baby bottles had reliably detectable EA (22–95% RME2) when extracted in either saline or EtOH, including the bottle, nipple, anticolic device, and liner (data not shown).

In fact, all BPA-replacement resins or products tested to date ($n > 25$) released chemicals having reliably detectable EA (data not shown), including polyethersulfone and PETG, sometimes having more total EA measured as %RME2 than many PC products when stressed. For example, the %RME2 released by various BPA-free baby and water bottle component parts extracted by saline or EtOH solutions and exposed to one or more common-use stresses can be greater than PC products under the same conditions (Figure 2). UV stress, in particular, often leads to the release of chemicals having greater EA than BPA-containing HC plastics currently sold. For example, saline extracts of BPA-free baby bottle B₃ (Figure 2) after exposure to UV showed greater EA than did any of the PC baby bottle extracts after any of the stresses. Saline extracts from BPA-free baby bottle B₁ after any of the stresses (microwave, autoclave, or UV) showed greater EA than did the saline extracts from PC baby bottle B₂ after any of the stresses. EtOH extracts from BPA-free baby bottle B₁ after UV stress showed greater EA than extracts from PC baby bottle B₁. Saline extracts from BPA-free baby bottle B₂ after microwave or autoclave stresses showed greater EA than did saline extracts from PC baby bottles B₁ or B₂ after any of the stresses. Note also in Figure 2 that multiple extracts of the same product using the same solvent/stress combination typically gave rather similar %RME2 data, but different solvent/stress combinations gave very different results, from very high EA to nondetectable EA. For example, EtOH extracts from PC baby bottle B₂

Figure 3. Properties of monomers and polymers used to make common resins.

Polymers	Monomers	Structures	EA	Toxicity ^a
Flexible polymers				
Low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), high density polyethylene (HDPE)	Ethylene		No	No
Polypropylene homopolymer (PPHO)	Propylene		No	No
HC polymers^b				
Copolymer using terephthalate PETG	1,4-Cyclohexanedimethanol, dimethyl terephthalate ^c		Yes ^d	No
Polycarbonate (PC)	Bisphenol A, ^e phosgene		Yes	Yes
Polyethylene terephthalate (PET)	Dimethyl terephthalate ^e		Yes ^d	No
Polystyrene (PS)	Styrene		Yes ^d	No
Polypropylene copolymer (PPCO)	Propylene, ethylene		No	No
Cyclic olefin polymer (COP), cyclic olefin copolymer (COC)	Ethylene, norbornene		No	No
Polyacrylonitrile (PAN)	Acrylonitrile		No	Yes
Polyethersulfone (PES)	1,4-bis(4-Chlorophenyl)sulfone, 1,4-dihydroxybenzene ^e		Yes ^d	No

^aPolymer exhibits other toxic effects (e.g., cellular damage or carcinogenicity), or toxic chemicals (e.g., phosgene and acrylonitrile) are used or produced during polymerization. ^bHC polymers generally have a glass transition temperature (T_g) above room temperature and limited or no ability to crystallize. ^cMonomer has anti-EA in MCF-7 assays. ^dUnder certain conditions, degradation products exhibit EA. ^eMonomer has EA in MCF-7 assays.

showed very high EA under all stress conditions, whereas saline extracts of the same bottle under the same stress conditions showed no detectable EA. Hence, to reliably detect EA, plastic resins or products must be extracted with both polar and nonpolar solvents and exposed to common-use stresses.

Discussion

Most plastic products release chemicals having EA. Our data show that both more polar (e.g., saline) and less polar (e.g., EtOH) solvents should be used to extract chemicals from plastics because the use of only one solvent significantly reduces the probability of detecting chemicals having EA. The ability to detect more polar and less polar chemicals having EA is important because plastic containers may hold either type of liquid or a liquid that is a mixture of more polar and less polar solvents (e.g., milk). When both more polar and less polar solvents are used, most newly purchased and unstressed plastic products release chemicals having reliably detectable EA independent of the type of resin used in their manufacture, type of product, processing method, retail source, and whether the product had contents before testing. However, the lack of significant difference in average percentage having detectable EA between plastic items with and without contents does not imply that the contents do not affect the total EA or specific chemicals having EA released by individual plastic items.

Our data show that most monomers and additives that are used to make many commercially available plastic items exhibit EA. Even when a "barefoot" polymer (no additives) such as PE or polyvinyl chloride does not exhibit EA, commercial resins and products from these polymers often release chemicals (almost certainly additives) having EA.

We found that exposure to one or more common-use stresses often increases the leaching of chemicals having EA. In fact, our data suggest that almost all commercially available plastic items would leach detectable amounts of chemicals having EA once such items are exposed to boiling water, sunlight (UV), and/or microwaving. Our findings are consistent with recently published reports that PET products release chemicals having EA (Wagner and Oehlmann 2009) and that different PET products leach different amounts of EA. For example, different PET products release different amounts of EA measured as %E2 or %RME2 [see Supplemental Material, Table 5C (doi:10.1289/ehp.1003220)], almost certainly because different PET copolymer manufacturers choose different monomers, additive packages, and synthetic processes to produce PET copolymer resins.

Our data are consistent with the hypotheses that the presence of a phenolic moiety

is the best predictor of whether a chemical exhibits EA and that benzene moieties often probably convert to phenolic moieties when the monomer and/or polymer is exposed to one or more manufacturing or common-use stresses. For example, although in theory most organophosphites (antioxidants commonly used with HPs to provide synergistic oxidation protection) in their unaltered state should not bind to ERs [see Supplemental Material, Table 1 (doi:10.1289/ehp.1003220)], organophosphites are hydrolytically unstable and often produce phenols when exposed to water (Kattas et al. 2000). Most organophosphite antioxidants we tested exhibited detectable EA (data not shown).

Likewise, various additives that are high-molecular-weight HPs do not have EA, but if exposed to moist heat they can undergo hydrolysis and produce lower-molecular-weight phenolics that have EA. Therefore, antioxidants and other additives should be tested for EA both in their original, unstressed form and after stressing. We can identify monomers and additives (antioxidants, clarifiers, slip agents, colorants, inks, etc.) having no detectable EA for use at all stages of manufacturing processes to make flexible nontransparent or HC plastic items that are EA free, even after exposure to common-use stresses. All of our data suggest that, when both are manufactured in comparable quantities, carefully formulated EA-free plastic products could have all the fit-for-use properties of current EA-releasing products at minimal additional cost.

BPA free is not EA free. Although most items listed in Tables 1–3 would not be expected to contain BPA, nevertheless almost all stressed plastic items tested leached chemicals having reliably detectable EA measured as %RME2 if extracted with both more polar and less polar solvents. In response to market and regulatory pressures, BPA-free PET or PETG resins and products have recently been introduced as replacements for PC resins. However, all such replacement resins and products tested to date release chemicals having EA (measured as %RME2), sometimes having more EA than BPA-containing PC resins or products, especially when stressed by UV light (Figure 2, Table 3). Monomer or polymer breakdown products that have EA account for some of this EA, but the rest of the measured EA is almost certainly due to release of additives having EA in BPA-free products, including the bottle and many component parts of baby bottles advertised as BPA free.

Avoiding a potential health problem. We recognize that we quantitatively measured EA relative to E2 (EC₅₀ or %RME2) using sensitive assay and extraction protocols. Furthermore, it is almost impossible to gauge how much EA anyone is exposed to, given

such unknowns as the number of chemicals having EA, their relative EA, their release rate under different conditions, and their metabolic degradation products or half-lives *in vivo*. In addition, the appropriate levels of EA in males versus females at different life stages are currently unknown. Nevertheless, *a) in vitro* data overwhelmingly show that exposures to chemicals having EA (often in very low doses) change the structure and function of many human cell types (Gray 2008); *b) many in vitro* and *in vivo* studies document in detail cellular/molecular/systemic mechanisms by which chemicals having EA produce changes in various cells, organs, and behaviors (Gray 2008); and *c) recent epidemiological studies* (Gray 2008; Koch and Calafat 2009; Meeker et al. 2009; Swan et al. 2005; Talsness et al. 2009; Thompson et al. 2009) strongly suggest that chemicals having EA produce measurable changes in the health of various human populations (e.g., on the offspring of mothers given diethylstilbestrol, or sperm counts in Danish males and other groups correlated with BPA levels in body tissues).

Many scientists believe that it is not appropriate to bet our health and that of future generations on an assumption that known cellular effects of chemicals having EA released from most plastics will have no severe adverse health effects (Gray 2008; Talsness et al. 2009; Thompson et al. 2009). Because we can identify existing, relatively inexpensive monomers and additives that do not exhibit EA, even when stressed, we believe that plastics having comparable physical properties but that do not release chemicals having detectable EA could be produced at minimal additional cost.

REFERENCES

- Begley T, Castle L, Feigenbaum A, Franz R, Hinrichs K, Lickly T, et al. 2005. Evaluation of migration models that might be used in support of regulations for food-contact plastics. *Food Addit Contam* 22:73–90.
- Begley TH, Dennison JL, Hollifield HC. 1990. Migration into food of polyethylene terephthalate (PET) cyclic oligomers from PET microwave packaging. *Food Addit Contam* 7:797–803.
- Burton K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 62:315–323.
- Della Seta D, Minder I, Belloni V, Aloisi AM, Dessi-Fulgheri F, Farabolini F. 2006. Pubertal exposure to estrogenic chemicals affects behavior in juvenile and adult male rats. *Horm Behav* 50:301–307.
- De Meulenaer B, Huyghebaert A. 2004. Packaging and other food contact material residues. In: *Handbook of Food Analysis*, Vol 2 (Nollet LML, ed). 2nd ed. New York:Marcel Dekker, 1297–1330.
- Gray J, ed. 2008. *State of the Evidence: The Connection between Breast Cancer and the Environment*. 5th ed. Breast Cancer Fund. Available: <http://www.breastcancerfund.org/assets/pdfs/publications/state-of-the-evidence-2010.pdf> [accessed 4 June 2011].
- Hewitt SC, Deroo BJ, Korach KS. 2005. Signal transduction: a new mediator for an old hormone? *Science* 307:1572–1573.
- ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2003. ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays.

- NIH Publication No. 03-4503. Available: http://iccvam.niehs.nih.gov/docs/endo_docs/edfinalrpt0503/edfinrpt.pdf [accessed 3 November 2010].
- ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2006. Addendum to ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. NIH Publication No. 03-4503. Available: http://iccvam.niehs.nih.gov/docs/endo_docs/EDAddendFinal.pdf [accessed 3 November 2010].
- Kabuto H, Amakawa M, Shishibori T. 2004. Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. *Life Sci* 74:2931–2940.
- Kattas L, Gastrock F, Levin I, Cacciatore A. 2000. Plastics additives. In: *Modern Plastics Handbook* (Harper CA, ed). 1st ed. New York:McGraw-Hill, 4.1–4.69.
- Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, et al. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a U.S. EPA-sponsored workshop. *Environ Health Perspect* (suppl 104):715–740.
- Koch HM, Calafat AM. 2009. Human body burdens of chemicals used in plastics manufacture. *Phil Trans R Soc B* 364:2063–2078.
- Leusch FDL, de Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al. 2010. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. *Environ Sci Technol* 44:3853–3860.
- Matsushima A, Teramoto T, Okada H, Liu X, Tokunaga T, Kakuta Y, et al. 2008. ERR γ tethers strongly bisphenol A and 4- α -cumylphenol in an induced-fit manner. *Biochem Biophys Res Commun* 373(3):408–413.
- Meeker JD, Sathyanarayana S, Swan SH. 2009. Phthalates and other additives in plastics: human exposure and associated health outcomes. *Philos Trans R Soc Lond B Biol Sci* 364:2097–2113.
- Natarajan N, Shambaugh GE III, Elseth KM, Haines GK, Radosevich JA. 1994. Adaptation of the diphenylamine (DPA) assay to a 96-well plate tissue culture format and comparison with the MTT assay. *BioTechniques* 17:166–171.
- National Research Council. 1999. *Hormonally Active Agents in the Environment*. Washington, DC:National Academies Press.
- Newbold RR, Jefferson WN, Padilla-Banks E, Haseman J. 2004. Developmental exposure to diethylstilbestrol (DES) alters uterine response to estrogens in prepubescent mice: low versus high dose effects. *Reprod Toxicol* 18:399–406.
- Patisaul HB, Fortino AE, Polston EK. 2006. Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. *Neurotoxicol Teratol* 28:111–118.
- Patisaul HB, Todd KL, Mickens JA, Adewale HB. 2009. Impact of neonatal exposure to the ER α agonist PPT, bisphenol-A or phytoestrogens on hypothalamic kisspeptin fiber density in male and female rats. *Neurotoxicology* 30:350–357.
- Plastics News. 2011. Resin Pricing Chart. 21 February: 21–22.
- Sax L. 2010. Polyethylene terephthalate may yield endocrine disruptors. *Environ Health Perspect* 118:445–448.
- Soto AM, Justicia H, Wray JW, Sonnenschein C. 1991. *p*-Nonyl-phenol: an estrogenic xenobiotic released from “modified” polystyrene. *Environ Health Perspect* 92:167–173.
- Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* 103(suppl 7):113–122.
- Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 113:1056–1061.
- Talsness CE, Andrade AJ, Kuriyama SN, Taylor JA, vom Saal FS. 2009. Components of plastic: experimental studies in animals and relevance for human health. *Philos Trans R Soc Lond B Biol Sci* 364:2079–2096.
- Thompson RC, Swan SH, Moore CJ, vom Saal FS. 2009. Our plastic age. *Philos Trans R Soc Lond B Biol Sci* 364:1973–1976.
- Till DE, Ehntholt DJ, Reid RC, Schwartz PS, Sidman KR, Schwoppe AD, et al. 1982. Migration of BHT antioxidant from high density polyethylene to foods and food simulants. *Ind Eng Chem Prod Res Dev* 21:106–113.
- vom Saal FS, Nagel SC, Timms BG, Welshons WV. 2005. Implications for human health of the extensive bisphenol A literature showing adverse effects at low doses. *Toxicology* 212:244–252.
- Wagner M, Oehlmann J. 2009. Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles. *Environ Sci Pollut Res* 16:278–286.

Références

En Gynécologie Obstétrique

Decades of discovery: the selective oestrogen receptor modulator (SERM) story:
The 2011 St. Gallen Prize.

V. Craig Jordan (Washington, USA)

Infertilité : savoir être patient... et impatient
H. Leridon (Paris, France)

Ovarian cysts and tumors of the fetus, child, adolescent and young adult (Part one).

A. Alchek, L. Deligdisch (New-York, USA)

La microchirurgie tubaire en 2010 : état des lieux.
D.K. Tran (Nice, France)

Reconstruction mammaire après mastectomie.

E. Delay, C. Ho Quoc, A. Scovola, G. Toussaint, M. Viber (Lyon, France)

Estetrol, the new natural estrogen for clinical use in women.

M. Visser, H.J.T. Coelingh Bennink (The Netherlands)

Étiologies et prise en charge de la sécheresse vaginale.

J. M. Bohlbot (Paris, France)

Apport des nouvelles techniques chirurgicales et de l'imagerie dans la prise en charge du cancer du col utérin avancé.

W. Gerych, C. Rannon-Haverlant, M. Kaemmerlen, P. Mathevet (Lyon, France)

Menopausal Hormonal Changes and Bone Loss.

J. Iqbal, L. Sun, M. Zaidi (New York, USA)

La naissance de l'enfant du double espoir : une nouvelle voie thérapeutique ou le catalyseur d'un mal typiquement français.

R. Frydman, F. Lannuzon (Clamart, France)

Oocyte donation:

the Institut Universitari Dexeus experience.

E. Chai, B. Corolen, L. Laine, M. Bouda, R. Tur, P. Nolasco Barri (Barcelona, Spain)

Une cause monogénique aux aménorrhées hypothalamiques ?

Th. Brue (Marseille, France)

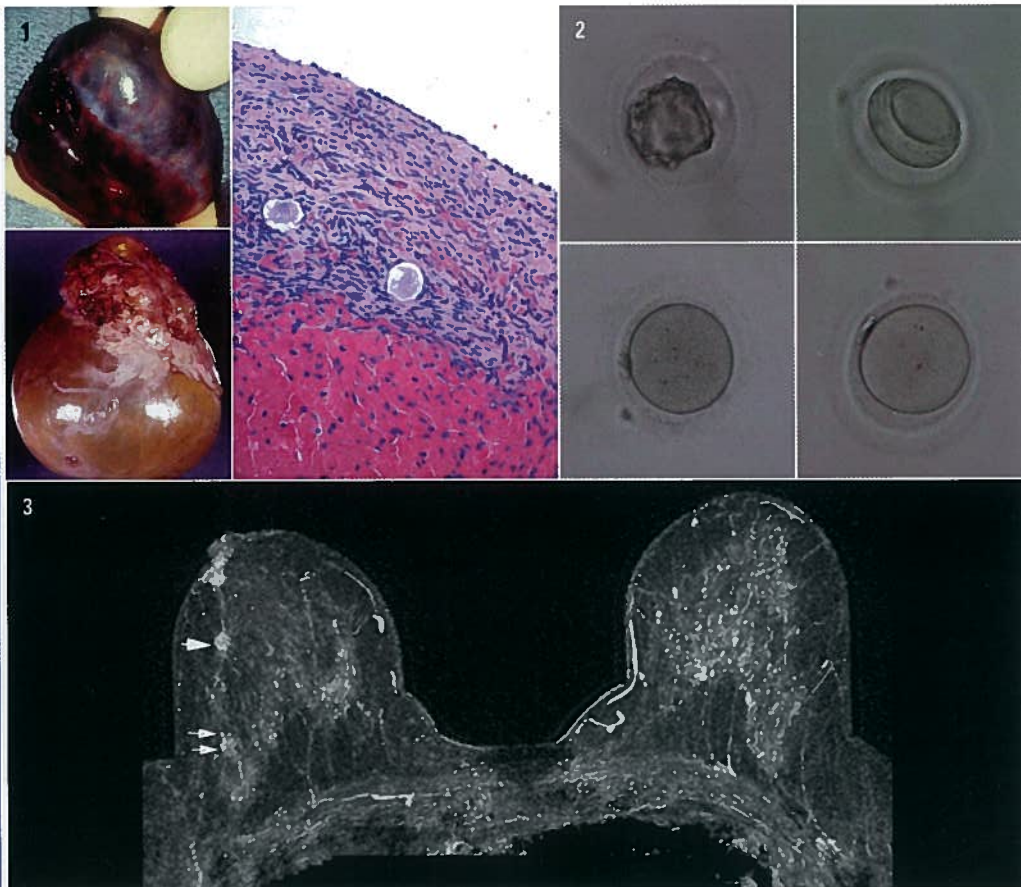
Tumorectomies multiples bilatérales grâce à l'imagerie multimodalités du sein : imprudence ou nouvelle tendance ?

K. Kinkel, P. Stuckelberger (Geneva, Switzerland)

12th St. Gallen International Breast Cancer Conference St. Gallen, Switzerland march 16-19, 2011.

How to remember the St. Gallen Consensus Conference 2011.

B. Pestalozzi (Zurich, Switzerland)

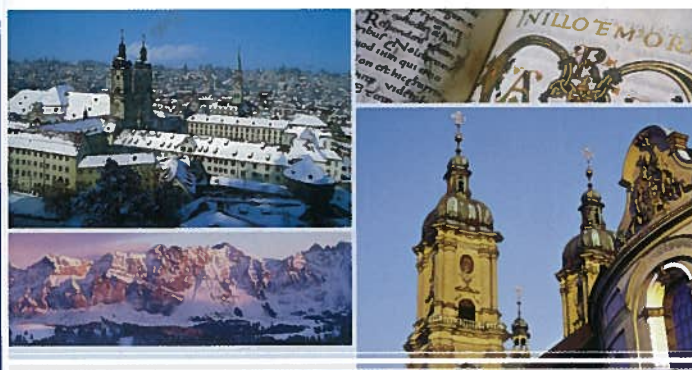


12th St.Gallen International Breast Cancer Conference

Primary Therapy of Early Breast Cancer
with Treatment Consensus Update

16 - 19 March 2011

St. Gallen, Switzerland



st.gallenoncology
primary therapy of early breast cancer

GMP
GENERAL MEDICINE PUBLICATIONS

ÉDITORIAL

Decades of Discovery: The Selective Estrogen Receptor Modulator (SERM) Story: The St. Gallen Prize

"I have but one lamp by which my feet are guided, and that is the lamp of experience. I know no way of judging of the future but by the past."
(Patrick Henry, the First Elected Governor of Virginia, 1775)

V. Craig Jordan*

Georgetown University Medical Center, 3970 Reservoir Rd NW, Research Building, Suite E501, Washington, DC 20057, Tel: 202.687.2897, Fax: 202.687.6402, E-mail: vcj2@georgetown.edu

Selective Estrogen Receptor Modulators (SERMs) are a well-established drug group in medicine. The SERMs are also unique, as their mechanisms depend on differentially switching on and switching off target sites around a woman's body – selectively [1]. Tamoxifen is the pioneering SERM [2] and the first medicine to be tested and approved for the reduction of risk of any cancer [3]. The problem to be solved was how to identify and treat the appropriate high risk women to reduce or eliminate their risk of developing breast cancer. The population based models [4] could focus down on a few thousand high risk women, but breast carcinogenesis would only be subverted in a few dozen.

These lucky few did not know who they were, amongst the thousands who were treated and who would never get breast cancer. Unfortunately, the strategy to apply the pioneering SERM, tamoxifen, for population based chemoprevention was flawed at the outset, as laboratory and clinical evidence predicted that there was an elevated risk of an increase in endometrial cancer for postmenopausal women [5-6]. This was a slight, but significant risk. Women worried. A range of other side effects (e.g. blood clots, cataracts, menopausal symptoms) would also be experienced by the many to benefit the few. The situation changed dramatically with the discovery that the two "lead" SERMs, tamoxifen and raloxifene, maintained bone density in laboratory animals, but also prevented mammary carcinogenesis [7-8]. Raloxifene was also less uterotrophic than tamoxifen. Would there be no endometrial risk? With the recognition of SERMs in the 1980's, a unique public health strategy was possible.

The new strategy was stated (twice) in the literature, which provided a simple roadmap for the pharmaceutical industry to follow (eventually!). *Are we looking in the wrong place? The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high-risk women to test "chemopreventive" agents.*

But are resources being used less than optimally? The problem is much greater than the current horizon. Indeed, even if we had the best chemopreventive for a minority of selected women, the overall impact on the disease might be negligible. An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, then a majority of women in general could be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions, then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone-dependent to hormone-independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century [9].

And subsequently: Is this the end of the possible applications for anti-estrogens? Certainly not! We have obtained valuable clinical information about this group of drugs that can be applied in other disease states.

Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer [10].

Today, new SERMs hold the promise of fulfilling the stated prediction from two decades ago. Lasofoxifene [11] for example, is approved in the E.U. for the prevention of osteoporosis in os-



* See biography page 392.

teopenic women, but at the same time, lasofoxifene reduces the incidence of breast cancer, coronary heart events, strokes and endometrial cancer. Raloxifene, the pioneering SERM to prevent both breast cancer and osteoporosis [12-13] is not as robust in its SERM pharmacology (there is no effect on coronary events or strokes) as lasofoxifene. Remarkably, lasofoxifene is 100 times more potent as a SERM; raloxifene is recommended at 60 mg daily but lasofoxifene is effective at 0.5 mg daily!

With this background of the current success of SERMs, my goal is to guide the reader through an evolution of ideas. History is often written as the achievement of Dynasties. But as with Dynasties, the dogma of the preceding Dynasty in medicine must be overcome, not by sudden force, but by unrelenting pressure and the reason of evidence. Only tenacity can change medicine through ideas as the standard of care is maintained and jealously guarded by the Dynasty.

My early catalytic role in the evolution of our story is well-documented in the refereed literature [2, 14]. Suffice to say as a pharmacologist, I had a passion to develop drugs to treat cancer. But where to start? By a series of accidents, I met the right people at the right time, but the career choice to study the pharmacology of nonsteroidal antiestrogen for my Ph.D. was then seen as a dead end. They were failed contraceptives and of only academic interest. But this was the point of a Ph.D. in Britain – training in research method with a Medical Research Scholarship. Thus, we enter the first of our 4 decades.

The 1970's: The re-invention of tamoxifen as the "gold standard" for the treatment and prevention of breast cancer

The Dynasty to be defeated in the opening years of the 1970's was combination cytotoxic chemotherapy. Chemotherapy was king, fresh from the victory over childhood leukemia and poised to "MOPP" up Hodgkin's Disease. It was reasoned by the Dynasty, if only the right combination of agents could be found in the lexicon of options, a cure was assured. No one was advocating antihormone (or as it was described, "hormone therapy"!) research and treatment.

I saw an opportunity to develop a failed contraceptive, ICI 46,474, further than was believed originally it could go. In 1972, ICI 46,474 was abandoned by the pharmaceutical industry for continuing clinical testing because there was no profit to be made. Nevertheless, the meeting between me and the Head of the Fertility Control Program, Arthur Walpole (or "Walop" as he was affectionately known), proved to be critical to our story. He examined my Ph.D. at Leeds University, but ensured I had the resources at the Worcester Foundation in Shrewsbury, MA, USA and Leeds University, to create a clinical strategy for this orphan drug. He ensured it was put on the market, now tell us how to use it! The strategy I conceived and implemented is in Fig. 1.

The strategy was based on 3 principles:
1) target the tumor ER with tamoxifen;

- 2) give long term adjuvant tamoxifen therapy;
- 3) plan for chemoprevention.

All these principles were unpopular at the outset, but persistence and hundreds of evidence-based lectures around the world to my clinical colleagues slowly defeated the Dynasty of combination cytotoxic chemotherapy to cure breast cancer. "Antihormone therapy" became the treatment of choice with long term adjuvant tamoxifen therapy targeted to the tumor ER (the first targeted therapy).

How bad was the first Decade of Discovery? If I may be so bold at this point to tell a story of my friend and colleague, Steven E. Jones, M.D. When I started my international journey to advocate my principles for adjuvant antihormone therapy, Steven was in Arizona, the co-Director of the Adjuvant Therapy of Cancer Meeting in the 1970's. I was setting up a Ludwig Institute in Bern, Switzerland and was invited to present my new ideas about the use of tamoxifen at their 1979 meeting. There I was, sandwiched between the greats of cytotoxic chemotherapy, Vince DeVita and Bernie Fisher. I, in contrast, was advocating a stealth attack on breast cancer with tamoxifen that by comparison had no side effects. Little hope, one would think, but the plan succeeded. Two decades later, Steven Jones rose at a meeting in Washington and started his talk by declaring, "Craig Jordan was correct." Through the clinical trials mechanism, it is now proven that long term (5 years) adjuvant tamoxifen treatment targeted to the tumor ER has enhanced the survival of millions of women worldwide [15]. An orphan drug that is cheap and easy to administer has and continues to save hundreds of thousands of lives annually.

Now our story changes to the second decade with the "new" fashion in oncology – chemoprevention.

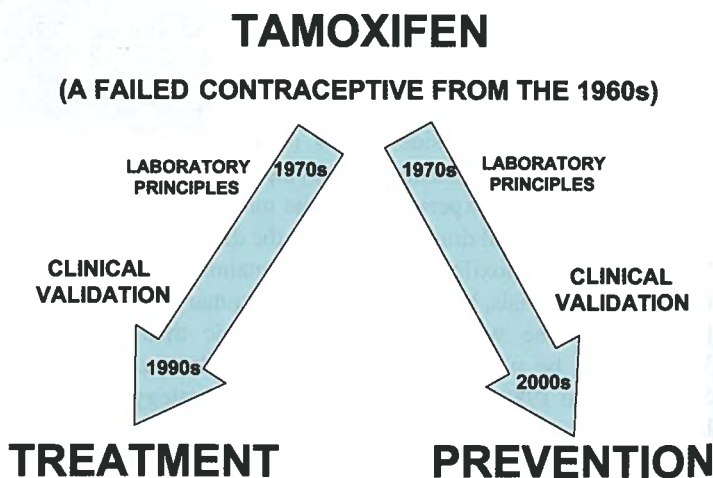


Fig. 1. The translational development of tamoxifen for breast cancer. Tamoxifen, was originally a failed oral contraceptive, ICI 46,474, that was abandoned by the pharmaceutical industry in 1972. The successful development of tamoxifen required three key components: 1) target the tumor ER with tamoxifen 2) give long term adjuvant tamoxifen therapy 3) plan for chemoprevention. As a result of targeting the tumor ER with tamoxifen, antihormone therapy became the treatment of choice for long term adjuvant tamoxifen therapy. This has been successfully validated through clinical trials [15] and in turn has saved the lives of millions of women around the world.

Table 1. Decades of Discovery

- The 1970's: The re-invention of tamoxifen as the "gold standard" for the treatment and prevention of breast cancer.
- The 1980's: SERMs surface.
- The 1990's: Raloxifene's Promise is a Reality.
- The 2000's: Estrogen-induced apoptosis?

The 1980's: SERMs surface

The idea of preventing cancer became popular in the 1980's. This is a noble goal and one of the primary goals of cancer research, but the goal has proved hard to address. The idea as applied to breast cancer has its origin with the French Scientist, Professor Antoine Lacassagne, who stated, at his lecture at the Annual Meeting of the American Association for Cancer Research in Boston (1936): "*If one accepts the consideration of adenocarcinoma of the breast as a consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer...*" [16]. However, Lacassagne's evidence was based on oophorectomy of mice from strains that develop a high incidence of mammary cancer and there were no mechanisms or compounds to advance and address the question. This would have to wait another quarter century with the serendipitous discovery of the nonsteroidal antiestrogens [10].

Tamoxifen was advanced for testing as a potential chemopreventive for breast cancer in the early 1980's based on three facts:

- 1) There was laboratory evidence that tamoxifen would prevent rat mammary carcinogenesis [17-19].
- 2) Tamoxifen was becoming widely used in medicine to treat breast cancer so, it was argued that side effects were known and anticipated. This was not really true, as it took translational research [5] to draw the attention of the clinical community of the small risk of endometrial cancer [6].
- 3) Tamoxifen, when used as an adjuvant therapy reduced the incidence of contralateral breast cancer [20].

Nevertheless, there was a major toxicological (and ethical issue) with treating well women with a drug classified as a "nonsteroidal antiestrogen" [21]. If, as was believed at the time, estrogen was good to build bone and to reduce the risk of coronary heart disease, what would be the value of the chemoprevention strategy that prevents breast cancer but condemns women to an elevated risk of crushing osteoporosis or fatal coronary heart disease. To address the concern, laboratory studies were initiated to evaluate the pharmacology of tamoxifen on estrogen target tissues.

Studies in rats demonstrated that both tamoxifen and the failed and discontinued breast cancer drug, raloxifene (then known as keoxifene) [22], both maintained bone density in ovariectomized rats [7] and prevented rat mammary carcinogenesis [8]. However, raloxifene was not as effective as tamoxifen, probably because of poor pharmacodynamics, i.e. raloxifene does not accumulate, is rapidly excreted and there is only 2% bioavailability by the oral route of administration [23]. This pharmacological fact was to

recur clinically following clinical trials 20 years later (see next section).

A pattern was emerging in the mid 1980's concerning the pharmacology of the nonsteroidal antiestrogens clomiphene, tamoxifen and raloxifene. The facts that lead to the SERM concept being described in my laboratory can now be summarized.

1) Clomiphene, a mixture of estrogenic *cis* and antiestrogenic *trans* geometric isomers, has bone preserving properties in the ovariectomized rat [24]. Clomiphene had been tested as a breast cancer drug in patients [25], but the manufacturer declined to advance development based on potential problems with cholesterol metabolism and a concern about cataracts. The drug remained the gold standard for the induction of ovulation where only five day courses were given [26].

2) The fact that clomiphene was an impure mixture of estrogenic and antiestrogenic isomers made the bone preserving effects uncertain. The estrogenic isomer might have been the favored pharmacologic agent at bone. In contrast, tamoxifen is the pure antiestrogenic *trans* isomer that preserves bone [7] and raloxifene is a fixed ring structure that is exclusively antiestrogenic (very weakly estrogenic) in the uterus, but estrogenic in bone [7].

3) Both tamoxifen and raloxifene are antitumor agents in rat mammary carcinogenesis [8].

4) Tamoxifen stimulates endometrial cancer growth (and mouse uterine growth) but blocks estradiol-stimulated growth of breast cancer transplanted in the same immune deficient animal [5, 27]. This experiment demonstrates target site specificity.

5) Tamoxifen lowers circulating cholesterol in the rat [28] and this property was included in the initial patent application which read: "*The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity*" [2].

The claims as a breast cancer drug were denied and required to be omitted in the United States until eventually a patent was awarded in 1985 in the Court of Appeals. In other words, tamoxifen was tested and marketed in America initially without patent protection for a dozen years. But, nobody cared, as there was little possibility of success, either as a therapy or commercially (or so everybody thought!).

Thus, based on all these data, primarily from my laboratory, the SERM concept surfaced and the roadmap for clinical development started as noted previously [9-10]. These data were the scientific basis of the Wisconsin Tamoxifen Study initiated in the late 1980's to evaluate the pharmacology of tamoxifen on bone density and circulating cholesterol. It was the proven clinical translation of the tamoxifen (SERM) concept to preserve bone density [29] and lower circulating cholesterol [30-31] that awakened the sleeping pharmaceutical industry to develop raloxifene to prevent and treat osteoporosis in postmenopausal women. This started with the "magical" patenting in 1992 of raloxifene for this indication [22] and the publication of laboratory studies confirming my work on the SERM actions of raloxifene in rats [32].

The 1990's: Raloxifene's Promise is a Reality

During the 1990's, I transitioned from my focus on laboratory investigations with SERMs to a role of "scientific resource" for major clinical trials. I was invited by Eli Lilly to chair their Oncology Advisory Committee, which had responsibilities to adjudicate breast cancer detection in their initial osteoporosis trial, Multiple Outcomes with Raloxifene Evaluation (MORE). Subsequently, Dr. Norman Wolmark would invite me to be the scientific chair of the largest breast chemoprevention study – the Study of Tamoxifen and Raloxifene (STAR).

The MORE trial recruited 7,705 postmenopausal women with osteoporosis to be randomized to placebo, 60 or 120 mg raloxifene daily. Raloxifene reduced fractures of the spine by 40% over the initial 3 year evaluation period [12]. In our parallel evaluation of the incidence of breast cancer, there was a significant decrease in the incidence of ER-positive breast cancer by 70% with no increase in endometrial cancer [12]. The laboratory concept of SERMs [10] translated to the clinic. Women being treated for osteoporosis would develop less breast cancer if they took raloxifene. But here was an important pharmacological point – it was proved that they must keep taking raloxifene to obtain benefit. This laboratory principle noted with rapidly excreted SERMs in the 1980's [8, 18] was to emerge as a clinical fact from the STAR trial after treatment stopped (see later).

What happened to tamoxifen in chemoprevention, Professor Trevor Powles initiated the first pilot toxicity study of tamoxifen in high risk women in the early 1980's [33], but it was Dr. Bernard Fisher who successfully conducted the first randomized placebo controlled clinical chemoprevention trial of tamoxifen in women at high risk for breast cancer. All preclinical predictors were confirmed-tamoxifen reduced the incidence of breast cancer, increased the incidence of endometrial in postmenopausal women and there was a decrease, though not significant in fracture rate [3, 34]. Unanticipated information (though prior clinical studies suggested an effect) was an increase in operations for cataracts. The other fact consistent with the overview analysis of clinical trial for adjuvant therapeutic tamoxifen [15] was that tamoxifen alone caused a long term beneficial effect to suppress the development of breast cancer more than a decade after tamoxifen therapy stopped [35]. We will return to the science behind this observation later. The fact, as we noted, that tamoxifen increased endometrial cancer in postmenopausal women, now caused a turn to raloxifene, that had no increased endometrial cancer in MORE [12].

The STAR Trial pitted tamoxifen 20 mg daily against raloxifene 60 mg daily for 5 years to compare and contrast efficacy and side effects for the reduction of breast cancer incidence in high risk postmenopausal women. As an aside, I was often asked how I would feel if raloxifene was found to be superior to tamoxifen. Happy – as the science of both drugs came from my laboratory and both drugs had to be reinvented as useful medicine after being essentially discarded by industry: tamoxifen, a failed contraceptive and raloxifene, a failed breast cancer drug. The first analysis of the STAR Trial showed equivalent efficacy to reduce the incidence of breast cancer by 50% [13]. However, side effects were

reduced with raloxifene. In particular, there was less endometrial hyperplasia with raloxifene and fewer hysterectomies. Operations for cataracts were lower on raloxifene. This analysis was conducted during raloxifene therapy [13], but the subsequent analysis conducted after therapy had stopped [36] demonstrated tamoxifen had a sustained antitumor action whereas there was a reduced (75%) efficacy for raloxifene. The drugs were different with their pharmacology and raloxifene must be given indefinitely.

We conclude that the fact that raloxifene is a drug with low bioavailability and therefore the pharmacodynamics to concentrate at the target site – the effect on the breast tissue is reduced. If sustained, local concentrations of tamoxifen and raloxifene are different and the elevated concentrations of tamoxifen drive cell population to evolve differently than those exposed to low levels of raloxifene, there will be, therefore, consequences for tumorigenesis and the evolution of drug resistance. We hypothesize that the low levels of raloxifene remain therapeutically "antiestrogenic" for the duration of therapy, but the endogenous estrogen from the woman's own body causes nascent tumor regrowth.

In contrast, the sustained high concentrations of tamoxifen locally in the breast causes a change in the evolution of the breast cancer cell population that in some way leaves an "antitumor memory" for years after therapy stops – but how? This leads us to the final decade of discovery: estrogen-induced apoptosis.

The 2000's: Estrogen-induced apoptosis?

The first chemical therapy to treat any cancer successfully was the use of high dose estrogen therapy to treat metastatic breast cancer in postmenopausal patients [37]. High dose estrogen therapy became the standard of care until the introduction of tamoxifen in the 1970's [10, 38]. At the end of his career, Sir Alexander Haddow FRS, presented the inaugural Karmofsky Lecture, where he expressed his disappointment about the lack of progress in understanding mechanisms: "...the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us..." [39]. What was known was that the high dose estrogen therapy was more effective as a breast cancer treatment the further away the patient was from the menopause, but why?

The advance in our understanding was to await an examination of model systems in the laboratory to decipher the mechanisms of antihormone drug resistance (Fig. 2). The whole topic has recently been summarized [40], but the facts must be stated to illustrate how transparency in nature can occur through unanticipated results in another area of research.

The first transplantable model of resistance to long term tamoxifen therapy demonstrated unique qualities. Acquired resistance is evidenced by tamoxifen-stimulated (actually SERM-stimulated as it turns out) growth. Tumors grow because of tamoxifen, not in spite of tamoxifen, as occurs with all other anticancer agents. What was even more surprising was the fact that when tamoxifen treatment is stopped, then estrogen again can

NEW CONCEPT EVOLUTION OF SERM RESISTANCE

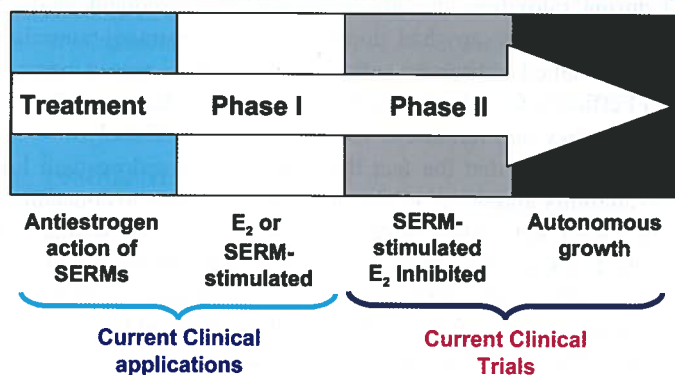


Fig. 2. The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The pure antiestrogen, fulvestrant, destroys the ER and prevents tumor growth in SERM-resistant disease. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of estrogen. These distinct phases of laboratory drug resistance have their clinical parallels and this new knowledge is being integrated into the treatment plan.

stimulate growth. This model replicates tamoxifen resistance during the treatment of ER-positive metastatic breast cancer: resistance occurs within a couple of years, estrogen or tamoxifen is required for continued growth and estrogen withdrawal or fulvestrant (the pure antiestrogen that causes destruction of the ER) is an appropriate second line therapy. What the model of acquired resistance did not do was explain how it was possible to use 5 years of adjuvant tamoxifen therapy to treat patients selectively. If the laboratory model was correct, and had been available at the time long term adjuvant therapy was planned as a treatment strategy, then no one would consider treatment longer than a year for adjuvant antihormone therapy. It would obviously be dangerous for patients. The same argument was used in the 1970's by the clinical community. Tamoxifen cannot control metastatic breast cancer on average, more than 2 years, so one cannot give long term (greater than 5 years) adjuvant tamoxifen. We were missing something fundamental about the biology of micrometastatic breast cancer exposed to long term tamoxifen therapy.

The breakthrough in understanding came through serendipity and as always, with outstanding graduate students with exceptional laboratory skills. The model of acquired resistance to tamoxifen could only only be maintained by serial transplantation in successive generations of tamoxifen-treated athymic mice. We were unable to transfer the tumors to cell culture for study, so the expense of preserving the only naturally developed model of resistance to tamoxifen had to be born. That, as it turned out, was the good, new and an opportunity for future discovery.

The acquired drug resistance to tamoxifen evolves in an environment of tamoxifen (Fig. 2). Retransplantation of tumor into

further tamoxifen treated mice causes adapted cell populations to develop rather than rely entirely on tamoxifen for growth (Phase I), but as the survival networks become reconfigured, a vulnerability emerges (Phase II). After 5 years of exposure to tamoxifen, the resulting tumor no longer sees estrogen as a survival signal, but as an apoptotic trigger (Phase II).

In 1992, these data were presented for the first time at the St. Gallen Breast Cancer Conference [41]. The hypothesis advanced was that the termination of tamoxifen, at the correct time, was important for the woman's own estrogen to destroy the microfoci of appropriately prepared target cells. This new biology of physiologic estrogen causing apoptosis was the reason for the enhanced survivorship of patients treated with a full 5 years of tamoxifen. As a result, a shift in thinking occurred and the clinical trials community subsequently exploited the concept, now published in the refereed literature [42], that therapeutic estrogen or indeed "physiologic estrogen" in the form of low dose estrogen replacement therapy (ERT) could cause the correctly configured tumors with acquired antihormone resistance to regress [43-44]. The Estrogen Dynasty originally deposed, struck back. Nature answered as well. Today, there is much interest in the paradoxical actions of physiologic estradiol in breast cancer [45-46]. Recent results from the Women's Health Initiative demonstrate a reduction in the incidence of breast cancer for hysterectomized, postmenopausal women who take long term estrogen replacement therapy [47]. Like our tamoxifen story, the effects persist for years following stopping ERT [48]. Practical advances, not only in the therapy of cancer, but preemptively in "natural" chemoprevention may result from these findings in the future.

In summary, I have mapped out the Decades of Discovery that emerged from a single quest some 40 years ago – to develop a drug useful for the treatment and prevention of breast cancer. At the time, I could count on the fingers of one hand, the people who were interested in the quest. No one cared, and it was not going to happen. But science is not like that; as in politics, ideas have their time but it is really about people and a passion to keep the flame of truth alight. I am immensely grateful to Professor Hans-Jörg Senn and his Committee for selecting me to receive the St. Gallen Prize for Advances in Breast Cancer Research. Thanks also go to my friends and colleagues Aron Goldhirsch and Richard Gelber. We all started our personal journeys together in Bern, Switzerland in the late 1970's and we remain older friends and colleagues to this day. Most importantly, I thank the 40 years of "Tamoxifen Teams" that worked and trained with me in my laboratories in Leeds University (UK), WFEB (US), Ludwig Institute for Cancer Research, Bern (Switzerland), University of Wisconsin (Madison), Northwestern University (Chicago), Fox Chase Cancer Center (Philadelphia) and the Lombardi Comprehensive Cancer Center (Washington, DC). I had the privilege to guide their lives and they turned the ideas we conceived into lives saved around the world.

The quotation in the heading of this Editorial was the one I used to open my Prize Lecture in St. Gallen, 16 March 2011: *"I have but one lamp by which my feet are guided, and that is the*

lamp of experience. I know no way of judging of the future but by the past" (Patrick Henry, the First Elected Governor of Virginia, 1775). My lamp was tamoxifen. However, this journey, as I hope I have illustrated, is so much more than the successful development of tamoxifen for the adjuvant treatment of breast cancer. It is about a way of constructing a conversation with nature with the goal of defeating a powerful enemy within us – cancer.

Acknowledgement

Grant support (to VCJ): This work (VCJ) was supported by the Department of Defense Breast Program under Award number BC050277 Center of Excellence (this interdisciplinary research grant supports research into estrogen-induced apoptosis in breast cancer); subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008 from the National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

Références

- Jordan VC (2001) Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 61: 5683-7
- Jordan VC (2003) Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2: 205-13
- Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 90: 1371-88
- Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, Mulvihill JJ (1989) Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 81: 1879-86
- Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48: 812-5
- Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, Skoog L, Somell A, Theve T, Wilking N, et al. (1989) Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1: 117-20
- Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10: 31-5
- Gottardis MM, Jordan VC (1987) Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47: 4020-4
- Jordan VC (1988) Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 6: 589-95
- Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 50: 4177-89
- Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, Goldstein S, Sriram U, Lee A, Thompson J, Armstrong RA, Thompson DD, Powles T, Zanchetta J, Kendler D, Neven P, Eastell R (2010) Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med* 362: 686-96
- Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281: 2189-97
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Jr., Wade JL, 3rd, Robidoux A, Margoless RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295: 2727-41
- Jordan VC (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 147 (Suppl 1): S269-76
- EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 365: 1687-717
- Lacassagne A (1936) Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer* 27: 217-25
- Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer* 12: 419-24
- Jordan VC, Allen KE (1980) Evaluation of the antitumor activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 16: 239-51
- Jordan VC, Allen KE, Dix CJ (1980) Pharmacology of tamoxifen in laboratory animals. *Cancer Treat Rep* 64: 745-59
- Cuzick J, Baum M (1985) Tamoxifen and contralateral breast cancer. *Lancet* 2: 282
- Jordan VC (1984) Biochemical pharmacology of antiestrogen action. *Pharmacol Rev* 36: 245-76
- Lewis JS, Jordan VC (2006) Case Histories: Raloxifene. *Comprehensive Medicinal Chemistry II* 8: 103-21
- Snyder KR, Sparano N, Malinowski JM (2000) Raloxifene hydrochloride. *Am J Health Syst Pharm* 57: 1669-75; quiz 76-8
- Beall PT, Misra LK, Young RL, Spjut HJ, Evans HJ, LeBlanc A (1984) Clomiphene protects against osteoporosis in the mature ovariectomized rat. *Calcif Tissue Int* 36: 123-5
- Herbst AL, Griffiths CT, Kistner RW (1964) Clomiphene Citrate (Nsc-35770) in Disseminated Mammary Carcinoma. *Cancer Chemother Rep* 43: 39-41
- ASRM PC (2006) Use of clomiphene citrate in women. *Fertil Steril* 86: S187-93
- Jordan VC, Robinson SP (1987) Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 46: 1870-4
- Harper MJ, Walpole AL (1967) A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil* 13: 101-19
- Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 326: 852-6
- Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, Feyzi J, DeMets DL (1991) Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 115: 860-4
- Love RR, Newcomb PA, Wiebe DA, Surawicz TS, Jordan VC, Carbone PP, DeMets DL (1990) Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst* 82: 1327-32
- Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, Cullinan GJ, Bendele R, Kauffman RF, Bensch WR, et al. (1994) Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. *J Clin Invest* 93: 63-9
- Powles TJ, Hardy JR, Ashley SE, Farrington GM, Cosgrove D, Davey JB, Dowsett M, McKinna JA, Nash AG, Sinnett HD, et al. (1989) A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer* 60: 126-31
- Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margoless RG, Runowicz CD, James JM, Ford LG, Wolmark N (2005) Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 97: 1652-62

35. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M (2007) Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 99: 283-90
36. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, 3rd, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N (2010) Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 3: 696-706
37. Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of Synthetic Oestrogens on Advanced Malignant Disease. *Br Med J* 2: 393-8
38. Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 69: 1243-54
39. Haddow A (1970) Thoughts on Chemical Therapy. *Cancer* 26: 737-54
40. Jordan VC, Ford LG (2011) Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-Induced Apoptosis. *Cancer Prev Res (Phila)*: Epub (Ahead of print)
41. Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 127: 23-33
42. Yao K, Lee ES, Bentrem DJ, England G, Schafer JJ, O'Regan RM, Jordan VC (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6: 2028-36
43. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 67: 111-6
44. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 302: 774-80
45. Lewis-Wambi JS, Jordan VC (2009) Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 11: 206
46. Maximov PY, Lewis-Wambi JS, Jordan VC (2009) The Paradox of Oestradiol-Induced Breast Cancer Cell Growth and Apoptosis. *Curr Signal Transduct Ther* 4: 88-102
47. Stefanick ML, Anderson GL, Margolis KL, Hendrix SL, Rodabough RJ, Paskett ED, Lane DS, Hubbell FA, Assaf AR, Sarto GE, Schenken RS, Yasmeen S, Lessin L, Chlebowski RT (2006) Effects of conjugated equine estrogens on breast cancer and mammography screening in postmenopausal women with hysterectomy. *JAMA* 295: 1647-57
48. LaCroix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD, Howard BV, Lewis CE, Wactawski-Wende J (2011) Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 305: 1305-14

Biography

V. Craig Jordan OBE, PhD, DSc, FMedSci, is the Scientific Director at the Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC, USA. He is the Vincent T. Lombardi Professor of Translational Cancer Research, and Professor of Pharmacology and Oncology at the Georgetown University Medical Center. Previously, he was the Diana, Princess of Wales Professor of Cancer Research at Northwestern University, Chicago IL (1999-2004) and the Alfred G. Knudson Professor of Cancer Research at the Fox Chase Cancer Center, Philadelphia PA (2004-2009). He was the Director of the Breast Cancer Research and Treatment Program at the Wisconsin Comprehensive Cancer Center in Madison WI (1987-1992), Director of the Lynn Sage Breast Cancer Research Program at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University (1993-2004), and Vice President and Research Director of Medical Science at the Fox Chase Cancer Center (2004-2009).

Jordan's four decades of translational research described the scientific principles to be used for the effective use of long term adjuvant tamoxifen and the laboratory basis for the use of tamoxifen as a chemopreventive. He first described the concept of selective oestrogen receptor modulation and the so-called SERMs are now a well-established drug group for the treatment and prevention of osteoporosis and breast cancer. He did the initial work on raloxifene in the laboratory, and helped to guide it through pivotal clinical trials. He was the Scientific Chair of the Study of Tamoxifen and Raloxifene (STAR). For more than twenty years, he has developed and studied the evolution of drug resistance to SERMs and aromatase inhibitors and his current work on the apoptotic actions of physiological oestrogen in antihormone resistant breast cancer is finding clinical applications.

Jordan has received international recognition for his pioneering studies in the adjuvant treatment and prevention of breast cancer, and his major prizes include the Karnofsky Award (2008), the American Cancer Society Chemoprevention Award (2006), the Charles F. Kettering Prize (2003), the American Cancer Society Medal of Honor (2002), the Bristol Myers Squibb Award (2002), and the European Institute of Oncology Breast Cancer Therapy Award (2001). In 2002, her Majesty the Queen appointed him Officer of the Most Excellent Order of the British Empire for his contributions to International Breast Cancer Research. His contributions to science and medicine have been recognized by prestigious professional societies, with election to the National Academy of Sciences (2009) in the United States, Fellowship of the Academy of Medical Sciences (2009) in the United Kingdom, and an honorary Fellowship of the Royal Society of Medicine and the Jephcott Gold Medal (2008). The St. Gallen Prize for Clinical Breast Cancer Research (2011).



Review

The St. Gallen Prize Lecture 2011: Evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities

V. Craig Jordan^{a,*}, Ifeyinwa Obiorah^a, Ping Fan^a, Helen R. Kim^a, Eric Ariazi^b, Heather Cunliffe^c, Hiltrud Brauch^d

^a Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D.C. 20057, USA

^b Fox Chase Cancer Center, Philadelphia, PA, 19111, USA

^c Translational Genomics, Phoenix, AZ, 85004, USA

^d Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany

ARTICLE INFO

Keywords:

Tamoxifen

Selective oestrogen receptor modulators (SERMs)

Raloxifene

Apoptosis

Oestrogen

Acquired drug resistance

Chemoprevention

SUMMARY

The successful translation of the scientific principles of targeting the breast tumour oestrogen receptor (ER) with the nonsteroidal anti-oestrogen tamoxifen and using extended durations (at least 5 years) of adjuvant therapy, dramatically increased patient survivorship and significantly enhanced a drop in national mortality rates from breast cancer. The principles are the same for the validation of aromatase inhibitors to treat post-menopausal patients but tamoxifen remains a cheap, life-saving medicine for the pre-menopausal patient. Results from the Oxford Overview Analysis illustrate the scientific principle of "longer is better" for adjuvant therapy in pre-menopausal patients. One year of adjuvant therapy is ineffective at preventing disease recurrence or reducing mortality, whereas five years of adjuvant tamoxifen reduces recurrence by 50% which is maintained for a further ten years after treatment stops. Mortality is reduced but the magnitude continues to increase to 30% over a 15-year period. With this clinical database, it is now possible to implement simple solutions to enhance survivorship. Compliance with long-term anti-hormone adjuvant therapy is critical. In this regard, the use of selective serotonin reuptake inhibitors (SSRIs) to reduce severe menopausal side effects may be inappropriate. It is known that SSRIs block the CYP2D6 enzyme that metabolically activates tamoxifen to its potent anti-oestrogenic metabolite, endoxifen. The selective norepinephrine reuptake inhibitor, venlafaxine, does not block CYP2D6, and may be a better choice. Nevertheless, even with perfect compliance, the relentless drive of the breast cancer cell to acquire resistance to therapy persists. The clinical application of long-term anti-hormonal therapy for the early treatment and prevention of breast cancer, focused laboratory research on the discovery of mechanisms involved in acquired anti-hormone resistance. Decades of laboratory study to reproduce clinical experience described not only the unique mechanism of selective ER modulator (SERM)-stimulated breast cancer growth, but also a new apoptotic biology of oestradiol action in breast cancer, following 5 years of anti-hormonal treatment. Oestradiol-induced apoptotic therapy is currently shown to be successful for the short-term treatment of metastatic ER positive breast cancer following exhaustive treatment with anti-hormones. The "oestrogen purge" concept is now being integrated into trials of long-term adjuvant anti-hormone therapy. The Study of Letrozole Extension (SOLE) trial employs "anti-hormonal drug holidays" so that a woman's own oestrogen may periodically purge and kill the nascent sensitized breast cancer cells that are developing. This is the translation of an idea first proposed at the 1992 St. Gallen Conference. Although tamoxifen is the first successful targeted therapy in cancer, the pioneering medicine is more than that. A study of the pharmacology of tamoxifen opened the door for a pioneering application in cancer chemoprevention and created a new drug group: the SERMs, with group members (raloxifene and lasofoxifene) approved for the treatment and prevention of osteoporosis with a simultaneous reduction of breast cancer risk. Thus, the combined strategies of long-term anti-hormone adjuvant therapy, targeted to the breast tumour ER, coupled with the expanding use of SERMs to prevent osteoporosis and prevent breast cancer as a beneficial side effect, have advanced patient survivorship significantly and promise to reduce breast cancer incidence.

© 2011 Elsevier Ltd. All rights reserved.

* Corresponding author. V. Craig Jordan, OBE, PhD, DSc, FMedSci, Scientific Director, Lombardi Comprehensive Cancer Center, Georgetown University, 3970 Reservoir Road NW, Research Building E-501, Washington, D.C. 20057, USA.

E-mail address: vcj2@georgetown.edu (V.C. Jordan).

Introduction

Professor Hans-Joerg Senn asked me to cast light on future opportunities for improving adjuvant anti-hormone therapy that can be implemented or tested in clinical trial. This I will do, but first I will preface my remarks with a quote from Patrick Henry, the first elected Governor of Virginia, who said it best: *"I have but one lamp by which my feet are guided, and that is the lamp of experience. I know no way of judging of the future, but by the past."* In 1969, when I started my research on the pharmacology of non-steroidal anti-oestrogen, there was no tamoxifen (Fig. 1), only

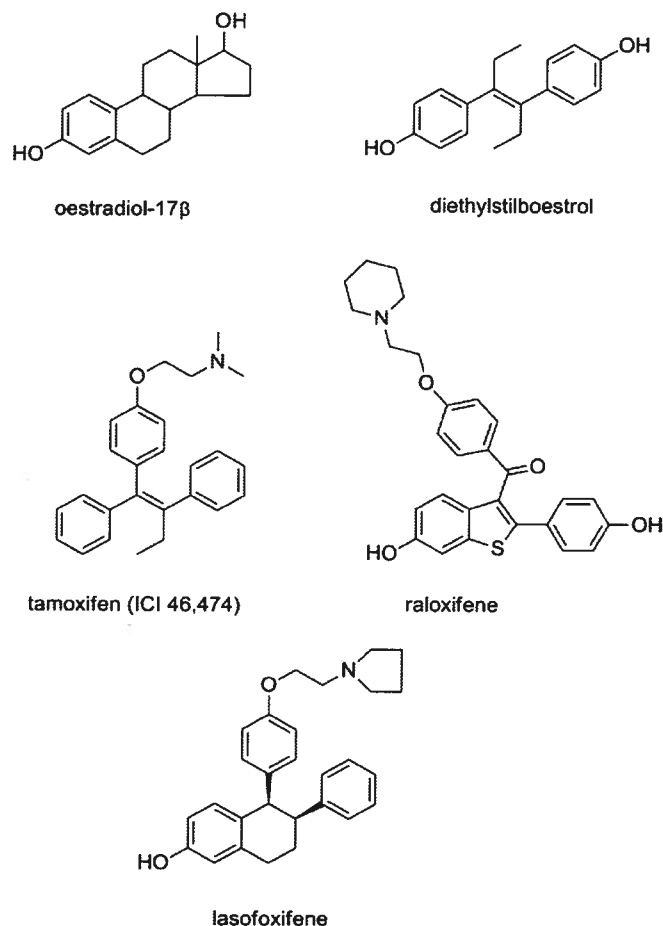


Fig. 1. The structure of medicines and compounds mentioned in the text. Oestradiol and diethylstilboestrol are oestrogens, whereas all others are selective oestrogen receptor modulators (SERMs) used in medicine for the treatment and chemoprevention of breast cancer (tamoxifen) or treatment and prevention of osteoporosis and the chemoprevention of breast cancer (raloxifene). The new SERM, lasofoxifene, is approved for the treatment and prevention of osteoporosis in the European Union.

ICI 46,474, an effective anti-fertility agent in rats.¹ The compound had anti-oestrogenic properties, so I proposed² to enhance its clinical application from an orphaned drug, with modest efficacy in metastatic breast cancer, to a targeted anti-cancer agent for adjuvant therapy and chemoprevention. Tamoxifen became my lamp, and subsequent laboratory research results shed light on the future of successful and safe adjuvant anti-hormone therapy, a new drug group of selected estrogen receptor modulation (SERMs),³ a lead compound in the SERMs raloxifene for clinical applications, the promise of multi-functional medicines, the unique qualities of acquired anti-hormone drug resistance and a new apoptotic biology of oestrogen in breast cancer (Fig. 1, Table 1).⁴ Tamoxifen, a failed contraceptive in women, is now a pioneering medicine in oncology¹ and is listed as an essential medicine by the World Health Organization.

The clinical validation^{5,6} of the laboratory principles of targeting the breast tumour oestrogen-receptor (ER)⁷ with long-term adjuvant antihormonal therapy (tamoxifen and oestrogen withdrawal)^{8,9} using a long acting anti-oestrogen, metabolically activated to potent hydroxylated metabolites,^{9–12} established a treatment strategy that continues to enhance the survivorship of millions of women world-wide. The key to success was the application of the first effective medicine to target the tumour through blocking oestrogen-stimulated growth at the ER, but coupled with the application of the counter-intuitive laboratory finding, that long-term adjuvant therapy would be superior to short-term therapy to control recurrence. The strategy succeeded, despite initial clinical findings that the tumour response to tamoxifen was not strongly correlated to ER status^{13,14} and the legitimate concern that long-term therapy would precipitate early drug resistance. This concern was based on the fact that tamoxifen was only an effective treatment in unselected metastatic disease for about a year or two,¹⁵ so why would extended or indefinite adjuvant tamoxifen treatment be effective at preventing recurrence in the adjuvant setting?

Clinical trials finally demonstrated that the laboratory principle of "longer was more effective at controlling recurrence" was correct.^{5,6} The subsequent development of the aromatase (AIs)¹⁶ expanded post-menopausal patient treatment options and reduced "oestrogen-like" side effects associated with tamoxifen, such as endometrial cancer and thromboembolic disorders.¹⁷ There was also a modest improvement of disease-free survival compared with tamoxifen. The widespread acceptance of long-term antihormonal therapy as the standard of care and the intense and exhaustive examination of patient population databases, now permit questions to be addressed to improve patient survivorship. At a time of shrinking resources for biomedical research but expanding menus of purported targeted drugs to close one pathway or another, it is time to apply simple, basic rules that will make an impact immediately on enhancing survivorship. Only then is it prudent to fine tune the results from a position of strength, by interrogating the tumour biology with blockers of survival pathways.

Table 1

Decades of translational discovery. The development of scientific principles in the laboratory were translated to clinical trials ten years later and subsequently became the standards for clinic care for the treatment or chemoprevention of breast cancer, or in the case of the SERM, raloxifene, a treatment option for the treatment and prevention of osteoporosis with the prevention of breast carcinogenesis as a beneficial side effect.

Decade	Scientific principle	Clinical benefit
1970s	Long-term adjuvant tamoxifen therapy targeted to ER	–
	Foundation of chemoprevention with tamoxifen	–
1980s	Selective ER modulation	Survival benefits for long-term adjuvant tamoxifen
1990s	Evolution of drug resistance to hormones	Chemoprevention with SERMs, tamoxifen and raloxifene
	Anti-tumour actions of physiologic oestrogens	
2000s	Oestrogen-induced apoptosis	Clinical translation of estrogen-induced apoptosis

Simple solutions to enhance survival

It seems obvious but it must be stated. The past 30 years of successful translational research is without value if an infrastructure does not exist to ensure that a patient's treatment is maintained when the medicine has proven value to aid survival from breast cancer. A medical team is available to support a patient's needs but there must be a refocus of the team to relearn basic principles: chronic therapy that requires years to provide benefit is worthless if the patients will not follow the regimen. This act will **dramatically** reduce their potential for survival. The fashion over the past four decades, for evidence based medicine, requires effective delivery. Significantly, delivery is a minor commitment compared to the effort behind discovering and proving the efficiency of a medicine in prospective clinical trials.

Based on the published evidence, several general principles are emerging about compliance. A recent analysis of anti-hormone therapy conducted in patients enrolled in the Kaiser Permanente of Northern California health system,¹⁸ revealed that approximately 30% of all patients discontinued either AI or tamoxifen early but of those who did continue, 70% were fully adherent for up to 5 years. Thus, only 49% overall are adherent for the full course of adjuvant anti-hormonal therapy. Predictors of non-adherence were African-American race, lumpectomy, unknown tumour site, lymph node involvement and other co-morbidities. Adherence was associated with Asian/Pacific Island ethnicity, married, earlier years of diagnosis (tamoxifen era), prior chemotherapy, radiation therapy and longer prescription refills. These and similar findings^{19,20} describe the extent of the problem but noncompliance with effective therapeutic agents also increases recurrence and mortality.^{21–23}

Another significant finding of the Hershman study¹⁸ was that young women under 40-years old were more likely to discontinue anti-hormone therapy. This group would be prescribed tamoxifen but reasons for stopping could be because the women chose to start a family or the menopausal side effects were too severe. In regard to the latter, many women have been routinely prescribed selective serotonin reuptake inhibitors (SSRIs) over the past decade to reduce menopausal side effects. Members of this drug group block the CYP2D6 enzyme that metabolically activates tamoxifen to the potent anti-oestrogen endoxifen, thereby (Fig. 2) impairing full drug benefit (Fig. 3).²⁴ However, it must be stressed that not all SSRIs

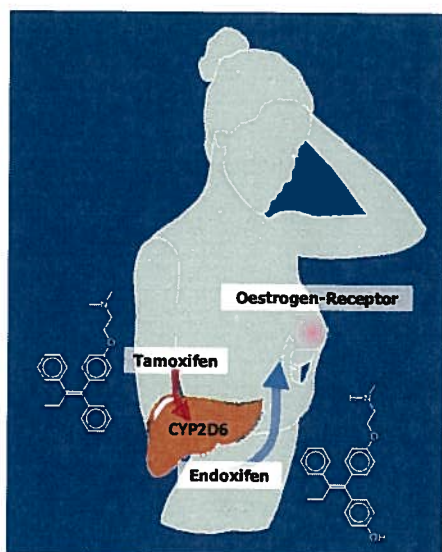


Fig. 2. The metabolic activation of tamoxifen with a low affinity to the tumour oestrogen receptor by the P₄₅₀ enzyme CYP2D6 enzyme to endoxifen with a high affinity for the tumour oestrogen receptor.

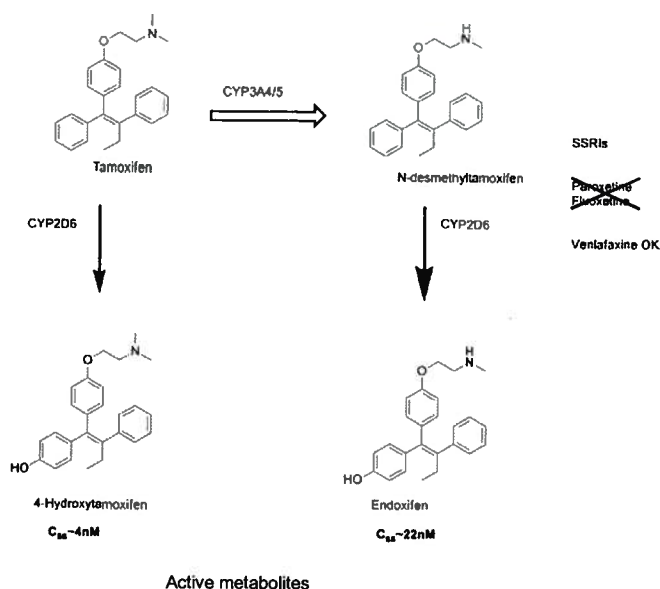


Fig. 3. The metabolism of tamoxifen to 4-hydroxytamoxifen, a metabolite with a high affinity for the oestrogen receptor. Tamoxifen's major metabolite is N-desmethyltamoxifen that has a similar binding affinity to the oestrogen receptors as tamoxifen. However, N-desmethyltamoxifen is metabolically activated to endoxifen, with a high binding affinity for the oestrogen receptor. The selective serotonin re-uptake inhibitors (SSRIs), paroxetine and fluoxetine block the metabolic activation of tamoxifen by blocking CYP2D6. Venlafaxine, a selective norepinephrine re-uptake inhibitor (SNRI), does not affect tamoxifen's metabolic activation, and therefore is the preferred choice to treat menopausal symptoms experienced with tamoxifen.

have the same ability to block tamoxifen metabolism and as a result, studies that group all SSRIs together are not uniformly consistent with the hypothesis.^{25,26} Nevertheless, the recent Canadian study of co-prescription of various SSRIs and the selective norepinephrine reuptake inhibitor (SNRI) venlafaxine does implicate paroxetine as increasing mortality during tamoxifen treatment and venlafaxine decreases mortality.²⁷ Overall, enhancing compliance and avoiding SSRIs that block CYP2D6 will significantly increase the chances of patient survival. That being said, the next issue to address is anti-hormone drug resistance.

Anti-hormonal drug resistance can be manifest in two forms for the ER positive tumour: intrinsic resistance where the tumour does not respond at all to anti-hormone therapy, despite being ER positive, and acquired anti-hormone therapy where the tumour initially responds to anti-hormone therapy but then grows despite the continuing treatment. Much effort has focused on an understanding of the molecular mechanism of intrinsic anti-hormone resistance and it seems that cross-talk between growth factor receptors and the low levels of ER have essentially made the ER irrelevant for cell survival. No scientific advance has yet reversed intrinsic resistance and aided patients. In contrast, there have been significant advances in understanding acquired anti-hormone resistance in the laboratory and these emerging data have been translated to clinical practice.

The challenge: acquired drug resistance

Clinical experience with the successful application of long-term tamoxifen as an adjuvant therapy produced a clear survival advantage for patients.²⁸ Unselected patients treated for 5 years with adjuvant tamoxifen lived longer than patients in the non-treatment (placebo) arm but who were treated with tamoxifen at first recurrence as they had metastatic breast cancer. The clinical results with successful adjuvant tamoxifen therapy demonstrated²⁸ that our understanding of the development of drug resistance to tamoxifen treatment in ER positive disease was incorrect on

July 25, 1987 (the publication date of the Scottish MRC trial), but supported the principle of early treatment of micrometastatic disease. Also, it highlights the fact that resistance to tamoxifen for the treatment of metastatic disease occurs rapidly within 2 years, and this biology did not apply to an adjuvant application of tamoxifen. Despite the fact that the rat mammary carcinoma model demonstrates that earlier, longer treatment with an anti-oestrogen was a suitable clinical strategy,⁸ there was no model of human diseases to test this hypothesis. However, in the mid-1980s, this was about to change. The ER positive breast cancer cell line MCF-7²⁹ exhibits oestradiol-stimulated tumor growth when transplanted into ovariectomized athymic mice. Tamoxifen blocks oestradiol-stimulated tumor growth but cannot maintain growth inhibition as ER positive tumors eventually grow despite tamoxifen treatment.³⁰ However, it seems that SERM and antihormonal resistance in breast cancer evolves and exposes a vulnerability in breast cancer that can be exploited in the clinic.³¹

The first transplantable model of tamoxifen resistance in breast cancer demonstrated that drug resistance to tamoxifen was unique.³² Although tamoxifen can initially block oestradiol-stimulated growth of MCF-7 cells, resistant ER positive tumors can use either oestradiol or tamoxifen to stimulate tumor growth (Fig. 4). Tumours do not grow unless treated with tamoxifen or oestradiol so in the ovariectomized mouse, this is equivalent to the “non-oestrogen state” created by aromatase inhibitors. Tumours also do not grow if treated with the pure anti-oestrogen fulvestrant that destroys the ER.^{33,34} This laboratory model replicates clinical experience with drug resistance to tamoxifen in metastatic breast cancer and explains why aromatase inhibitors or fulvestrant are effective second line treatments.^{35,36} So, how does a study of the drug resistance to tamoxifen in the laboratory explain the effectiveness of 5 years of adjuvant tamoxifen to reduce recurrence rates in ER positive breast cancer to tamoxifen by fifty percent and continue to reduce mortality a decade after tamoxifen treatment is stopped? The answer is the evolution and reconfiguration of cell survival pathways that occurs in micrometastatic breast cancer during years of treatment.

Continuous retransplantation of successive generations of tamoxifen-stimulated MCF-7 tumor lines into athymic mice for more than 5 years results in a derived tumor line that does not respond to physiologic oestradiol with growth but rapid tumor regression through apoptotic cell death (Fig. 5).^{37,38} These data were first presented at the St. Gallen meeting in 1992.³⁷ The concept offered at the time was that the ultimate and long lasting value of adjuvant tamoxifen therapy derives from stopping adjuvant tamoxifen when the woman's own oestrogen can now destroy the micrometastases that have been sensitized to

EVOLUTION OF SERM RESISTANCE

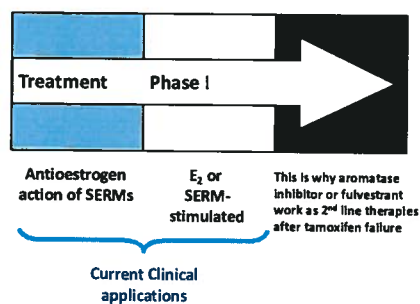


Fig. 4. The development of acquired antihormone resistance to selective oestrogen receptor modulators (SERMs) (tamoxifen or raloxifene). The unique feature of Phase I antihormone resistance is that oestrogen receptor positive breast tumours grow in response to either physiological oestradiol or the SERM. In the clinical setting (and laboratory models), an aromatase inhibitor (no oestrogen) or the pure anti-oestrogen, fulvestrant, that destroys the oestrogen receptor, stops the growth of Phase I resistant tumours to tamoxifen.³¹

St. Gallen 1992

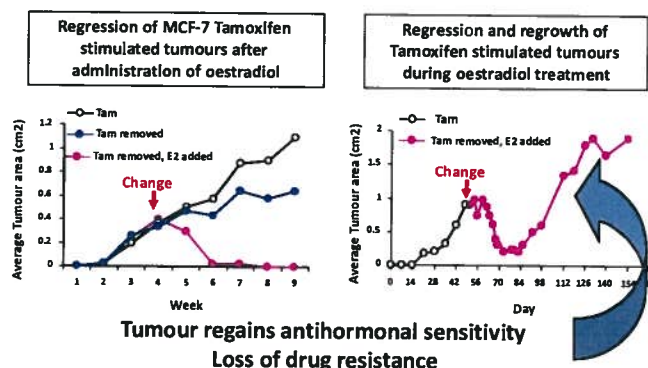


Fig. 5. Diagrammatic representation of the actions of physiologic oestradiol (E₂) on the growth of small phase II MCF-7 tamoxifen resistant tumours in ovariectomized athymic mice. A larger tumour will regress with oestradiol treatment but will eventually display oestrogen-stimulated growth. If tumours are re-transplanted into a new generation of ovariectomized athymic mice and treated with oestradiol, tamoxifen will block oestrogen-stimulated tumour growth.³⁸ First presented in St. Gallen, 1993.³⁷

oestrogen-induced apoptosis. The initial laboratory observations on low dose oestradiol-induced tumour regression were subsequently confirmed,³⁸ expanded^{39–42} and translated successfully to clinical trial.^{43,44} As a result, it is now possible to define the evolution of acquired anti-hormone therapy into a Treatment Phase where the anti-hormone blocks oestradiol stimulated tumour growth, Phase I when a SERM or oestradiol stimulates growth (or an aromatase inhibitor creates oestrogens independent growth) and Phase II when a SERM stimulates growth but physiological oestrogen provokes apoptosis either after stopping a SERM or after stopping an aromatase inhibitor (Fig. 6).

Thus, over the past four decades, general scientific principles have emerged and translated to clinical care for patients. The application of these principles of endocrine adjuvant therapy have benefited, and continued to benefit, millions of women worldwide, through a simple and cheap therapeutic intervention. We will now consider

NEW CONCEPT EVOLUTION OF SERM RESISTANCE

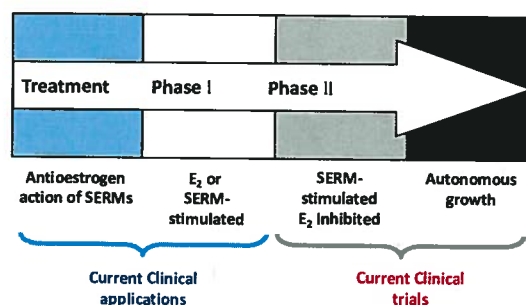


Fig. 6. The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumour growth. Tumours also continue to exploit oestrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumour growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of oestrogen. A similar evolution occurs with aromatase inhibitor resistance from oestrogen independent growth with a transition to oestrogen-induced apoptosis. These distinct phases of laboratory drug resistance have their clinical parallels and this new knowledge is being integrated into the treatment plan.

NF- κ B Non-canonical Pathway

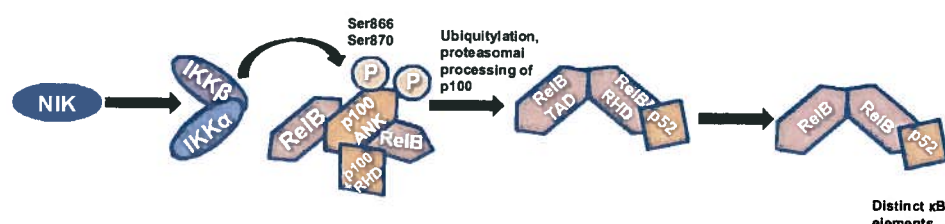


Fig. 7. The non-canonical pathway results in the activation of IKK α by NIK and phosphorylation of the NF- κ B subunit. This process results in the conversion of p100 to p52. It is the p52-RelB heterodimers that target distinct κ B elements on DNA. ANK: ankyrin-repeat motifs; NIK: NF- κ B kinase; RelB: NF- κ B family member; RHD: Rel-homology domain; TAD: transcriptional activation domain.

how emerging laboratory knowledge may reverse or at least hold Phase II resistance to enhance the longevity of the patient. We will, however then, revisit the clinical reality that increased tumour burden is a poor indicator of patient survival, so that the founding principles of our initial work, i.e. early treatment targeting the ER with long-term therapy² must be embraced by the clinical community.

Oestradiol-induced apoptosis under laboratory conditions

The administration of physiologic oestradiol to athymic mice implanted with phase II SERM (tamoxifen or raloxifene) resistant ER positive MCF-7 tumours^{38,40,41,45} causes tumours to stop growing and/or rapidly regress. Similarly, the long-term oestrogen deprived clinical cell line MCF-7:5C^{42,46} rapidly undergoes oestrogen-induced apoptosis both *in vitro* and *in vivo*. These laboratory observations are reminiscent of the pioneering studies of Sir Alexander Haddow FRS with his application of the first Chemical Therapy to successfully treat any cancer – high dose synthetic oestrogens to treat metastatic breast cancer.^{47,48} He observed a 25% response rate but these were short-lasting.⁴⁷ The observation was made that no responses were observed close to menopausal but often dramatic responses occurred in women in their late 60s and 70s. By 1970, during the presentation of the Inaugural Karnofsky Award Lecture at the American Society of Clinical Oncology (ASCO)⁴⁸ (incidentally, when I was starting my PhD in Pharmacology at Leeds University) he stated: "... the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us ...".

Now we know that the responses Haddow observed occur because of oestrogen deprivation following the menopause. Longer oestrogen withdrawal after menopause was more effective at creating Phase II resistance in select patients, but high dose oestrogen therapy was necessary. Based on laboratory studies and clinical correlations, anti-hormone therapy does a better job in driving the rapid evolution to Phase II resistance and as a result, only physiological oestrogen is necessary to trigger apoptosis. Haddow's paradox that stood for 40 years now has clarity and we can start to offer treatment options to exploit the concept further.

Cell culture models provide a vehicle to examine, over time, oestrogen-induced apoptosis with the aim of pharmacologic modulation and the discovery of mechanisms that may have relevance for patient care. Through a knowledge of mechanisms, the elegant oestrogen trigger for naturally initiating tumour cell death may subsequently be exploited to other treatment scenarios. If we can decipher the process of ER-induced apoptosis from its current obscurity, this knowledge could be applied with the discovery of new drugs to trigger the mechanism without the involvement of ER. The ER is our current guide and light to find a new drug group.

We have undertaken an extensive examination of the actions of oestradiol on the growth (MCF-7), immediate apoptosis (MCF-7:5C) and delayed apoptosis (MCF-7:2A)⁴⁹ of our model cells using a 2-week time course of gene activity documented through mRNA analysis, using Agilent Gene Arrays. These studies were conducted in collaboration with Dr. Eric Ariazi and Dr. Heather Cunliffe. We extensively analyzed the gene time course, and completed gene segregation based on hierarchical pathway analysis. We found that MCF-7 and MCF-7:2A, our control cells, remained quiescent during the initial few days of oestradiol treatment (1nM) whereas the pre-apoptotic MCF-7:5C cells responded with a massive rise in the activation of inflammatory genes. Analysis of the sequence of events during the first few days of gene activation, we propose that apoptosis occurs in MCF-7:5C cells by the exploitation of the non-canonical pathway for NF- κ B signal transduction (Fig. 7). Furthermore, we have mapped out the time-course activation of each caspase (except caspase 3 that is absent in MCF-7) and determined that caspase 4 is the first and controlling executioner to provoke programmed cell death. We have interrogated the apoptotic process with purported inhibitors of individual activated caspases to confirm our conclusion of the role of caspase 4. Blockade of caspase 4 blocks oestrogen-induced apoptosis.

Most importantly, the activation of inflammatory genes suggests that oestradiol-induced apoptosis could be inhibited or at least modulated by glucocorticoids. We have subsequently established that dexamethasone inhibits oestrogen-induced apoptosis in a concentration related manner. This novel observation may have important implications for the application of oestradiol-induced apoptosis for individualized patient care. Is it possible that the inadvertent administration of glucocorticoids during patient care could block oestrogen-induced apoptosis or that a patient's own glucocorticoids may also inhibit apoptosis, if patients are challenged with oestrogen following exhaustive anti-hormone therapy? The anti-glucocorticoid mifepristone (RU486) could potentially be used with oestrogen to block glucocorticoid action temporarily for a few weeks during low dose oestrogen administration to enhance apoptosis.

Examination of the Agilent gene array data confirmed our previous work⁴⁹ that elevated synthesis of glutathione is protecting MCF-7:2A cells from immediate apoptosis in response to oestrogen. Apoptosis appears to be retarded in MCF-7:2A cells but an activation of autophagy heralds an enhanced transcription of caspase 4 and then triggers oestrogen-induced apoptosis during the second week of oestradiol treatment. We have previously successfully used pharmacological inhibitors to test our hypothesis. Buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis,⁴⁹ enhances oestradiol-induced apoptosis from a slow event lasting 2 weeks to an immediate event. Unfortunately, BSO, though used extensively in clinical trial a decade or more ago, is no longer available to examine whether it is possible to enhance oestrogen-induced apoptosis in patients with select tumours.

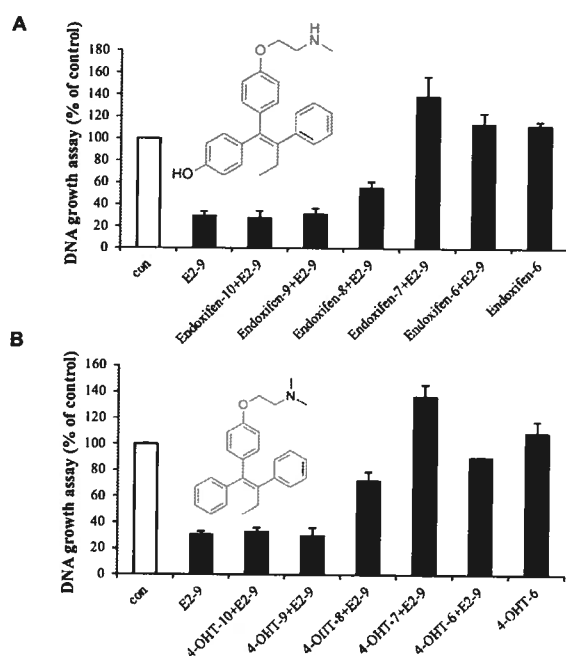


Fig. 8. The reversal of oestradiol-induced apoptosis (1nM) by increasing concentrations of 4-hydroxytamoxifen or endoxifen. This nonsteroidal anti-oestrogen effect highlights the ER dependence for oestradiol-induced apoptosis.

Thus far, our studies have described what happens, but the real question is how does the oestradiol/ER complex triggers apoptosis? Are there clues about the actual shape or structure of the oestrogen-ER complex that can be modulated and investigated further? The MCF-7:5C cells depend on a functioning ER for oestradiol-induced apoptosis. The pure anti-oestrogen fulvestrant binds to the ER and causes the rapid destruction of the protein complex. As a result, fulvestrant blocks oestradiol-induced apoptosis in a concentration related manner. Interestingly enough, the tamoxifen metabolites 4-hydroxytamoxifen (4OHTam) and endoxifen do not block or affect the autonomous growth of MCF-7:5C cells but do block the initiation of oestradiol-induced apoptosis. Herein lies a clue to the mechanism that triggers oestradiol-induced apoptosis (Fig. 8). X-Ray crystallographic studies of the ER ligand binding domain and the oestrogens, oestradiol and diethylstilboestrol (DES) and the SERMs 4OHTam⁵⁰ and raloxifene⁵¹ provide a fascinating insight into oestrogen and anti-oestrogen action. The solution of the crystal structures demonstrate that the planar oestrogens are sealed within the ligand binding domain by helix 12 which then allows co-activators to bind to the activating function (AF)-2 site on the complex. This event amplifies oestrogen action through gene transcription. In contrast, the bulky side chain of the triphenylethylene 4OHTam and the benzothiophene raloxifene prevent helix 12 from sealing the hydrophobic ligand binding domain which prevents coactivator binding to AF-2. The promiscuous oestrogen-like activity of 4OHTam is explained by the inability of the anti-oestrogenic side chain to neutralize and shield the exposed aspartate at position 351 at the surface of the ligand binding domain. This exposed carboxylic acid communicates with AF-1 to induce oestrogen-like actions. Raloxifene completely blocks and neutralizes the aspartate at 351 and the raloxifene-ER complex does not activate AF-1. This hypothesis has been successfully interrogated with changes in the ligand and the aspartate at 351 to modulate the activation of a model oestrogen target gene Transforming Growth Factor α .^{52–55} Overall, we concluded that activation of AF-1 by an exposed surface aspartate 351 confirms that helix 12 is not sealing the ligand binding domain so it can, therefore, communicate a signal to AF-1 to induce oestrogen-like gene activation. If aspartate 351 is masked under helix 12 with a planar

oestrogen then AF-2 is activated and the communication between AF-1 and aspartate 351 is mute. These data and conclusions subsequently resulted in a reclassification of oestrogens into class 1 (planar) and class 2 (non-planar)⁵⁶ using a simple assay to determine whether helix 12 was locking the ligand into the hydrophobic ligand binding domain or not. However, the biological significance of this molecular insight was not apparent until recently.

Based on the fact that 4OHTam blocks oestradiol-induced apoptosis at the ER and the statement that the "bulky side chain" of 4OHTam altered the conformation of the ER preventing helix 12 from sealing the ligand binding domain,⁵⁰ we advanced the hypothesis that the "bulky side chain" of 4OHTam was the phenyl ring of the oestrogenic triphenylbut-1-ene not just the *para*-dimethylaminoethoxy group traditionally associated with anti-oestrogen action. Perhaps the phenyl ring of the triphenylbut-1-ene anti-oestrogen was stopping helix 12 from sealing the binding site? A series of triphenylethylenes (TPEs), previously known to be classified exclusively as oestrogens in rodent uterine weight and vagina cornification assays, was used to establish oestrogenic activity in MCF-7 breast cancer cells. All compounds were found to be full oestrogens in growth assays compared with oestradiol and DES and to fully-activate an ERE luciferase report ER gene system in MCF-7 cells.⁵⁷ In contrast, while oestradiol and DES will trigger apoptosis and cell death in MCF-7:5C cells within a week, the synthetic TPE "oestrogens" do not provoke massive apoptosis and indeed block oestradiol-induced apoptosis. Studies using the CHIP assay at the ERE site in the promoter region of the oestrogen responsive pS₂ gene demonstrate that whereas oestradiol E₂ER complex is recruited with the co-activator SRC3 in AF-2 neither 4OHTam nor the TPE ER complexes are recruited to the promoter.⁵⁸

Overall, these data demonstrate that oestrogen-induced apoptosis is governed and programmed by the shape of the ER complex. As a consequence, shape governs coactivator binding at AF-2 and these events subsequently trigger apoptosis. A recent study⁵⁹ advances our initial oestrogen reclassification paper⁵⁶ and confirms, using a phage display library, that the shape of the ligand programs the external shape of the ER complex. A precise evaluation of the immediate early genes involved in the apoptotic response will describe the mechanism of the oestrogenic trigger for cell death. Exploitation of this knowledge may find applications in other disease states.

Oestrogen treatment: current clinical findings and translation to adjuvant therapy

The laboratory finding^{37–39} that acquired resistance to anti-hormone therapy evolves and exposes a vulnerability of breast cancer cells to the apoptotic actions of physiological oestrogen, provides an important insight into potential therapeutic applications. As previously noted in this paper, the anti-tumour effect of physiological oestrogen is reminiscent of the early therapeutic use of high-dose oestrogen therapy for the treatment of metastatic breast cancer in post-menopausal women.⁴⁷ It was noted that the further from menopause patients were, the more likely there was to be a tumour response, but these responses never exceeded 30% in any given population.

It is now clear that the acute oestrogen deprivation caused by anti-hormones speeds up the molecular adaptation and reconfiguration of vulnerable survival pathways. The surviving populations of susceptible breast cancer cells also have increased sensitivity to oestrogen-induced apoptosis. Low-dose oestrogen therapy now becomes a clinically viable strategy with the prospect of reducing oestrogen-associated side effects.

The laboratory data generated and published in the 1990s proposed the clinical strategy of using low-dose oestrogen therapy

Table 2

The proof of principle for (a) high-dose oestrogen (DES, 15 mg daily) triggering tumour responses in patients with metastatic breast cancer following exhaustive antihormone therapy⁴³ or (b) a comparison of high-dose oestrogen (oestradiol, 30 mg daily) or low-dose oestrogen (oestradiol, 6 mg daily), producing similar clinical benefit rates following the failure of therapy with an aromatase inhibitor.⁴⁴

(a) Response			
Complete	Partial	Stable disease	
4/32	6/32	2/32	

(b) Dose	No. of patients	Response	Clinical benefit
6 mg	34	10/34	29%
30 mg	32	9/32	28%

International Breast Cancer Study Group (IBCSG)

IBCSG 35-07 - Study Of Letrozole Extension (SOLE)

At completion of 4 to 6 years of prior adjuvant SERM/AI endocrine therapy, patients will be randomized to one of two treatment groups:

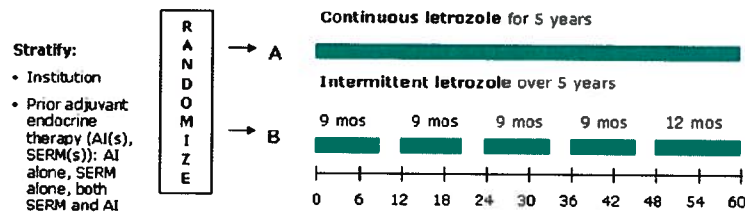


Fig. 9. The Schema for the Study of Letrozole Extension (SOLE) conducted by the International Breast Cancer Study Group (IBCSG 35-07). Patients randomized following five years of adjuvant antihormone therapy to letrozole continuously or intermittent letrozole (3-month drug holidays per year for 5 years). The rationale is that the woman's own oestrogen in the intermittent arm will trigger apoptosis in aromatase inhibitor resistant cells and reduce recurrence rates.

to "purge" breast cancer cells with Phase II-acquired anti-hormone resistance, but then the re-introduction of anti-hormone therapy would control oestradiol-stimulated tumour growth.^{37,38} A European trial led by Dr. Per Lonning⁴³ recruited patients with metastatic breast cancer following exhaustive anti-hormone therapy to determine the effect of treatment with standard high-dose DES (5 mg tid). Results are summarized in Table 2a. Select patients responded well with one patient subsequently reported⁶⁰ being disease-free more than 10 years after first initiating a high-dose oestrogen "purge" therapy. "... "

In a follow-up study, Ellis⁴⁴ compared and contrasted high-dose (10 mg tid) and low-dose (1 mg tid) oestradiol therapy in patients who relapsed during adjuvant aromatase inhibitor therapy. Results are summarized in Table 2b. Results were not as impressive as in the Lonning study probably because patients did not receive "exhaustive" endocrine therapy prior to an oestrogen "purge". Nevertheless, the clinical trial confirms that low-dose oestrogen can produce similar clinical benefit when compared with high-dose oestrogen treatment but with fewer serious side effects.

Finally, there is further clinical evidence from the Women's Health Initiative (WHI) that oestrogen replacement therapy (ERT) alone causes a decrease rather than increase in the incidence of breast cancer⁶¹, and a recent report from the Million Women Study in the UK demonstrates that oestrogen alone increases breast cancer incidence immediately following the menopause but if ERT is used more than 5 years after oestrogen exposure, oestrogen replacement therapy does not cause a rise in breast cancer incidence.⁶² An overarching explanation for these apparently confusing clinical observations is clarified by our evolving molecular model to exploiting the role of oestradiol in the life and death of breast cancer cells.^{63,64} We interpret these clinical findings based on the evolution of anti-hormone resistance as follows: breast cancer cells

in an environment of oestrogen only grow in response to exogenous oestrogen, but following long-term oestrogen deprivation surviving breast cancer cells either die or at least do not develop into tumours.

The clinical and laboratory database also provides continuing support for the ongoing adjuvant Study of Letrozole Extension (SOLE) trial (Fig. 9). Patients who have completed 5 years of adjuvant therapy with tamoxifen, an AI or any sequence are then randomized to an AI continuously for 5 years or an AI with a drug holiday for 3 months a year. The trial seeks to exploit the hypothesis, advanced at the 1992 St. Gallen Meeting, that a woman's own oestrogen may act as an anti-tumour agent after adjuvant anti-hormone therapy is stopped. The SOLE trial proposes a rigorous test of the hypothesis under controlled conditions that promises to create a practical advantage for patients following drug holidays. Results from this trial coupled with the expanding molecular database concerning the modulation of oestrogen-induced apoptosis may result in the proposition of regularly purging patients for a week or two with ERT if decades of anti-hormone therapy are to become common place in order that the disease is held in check and prevented from recurring. The question is now – at what point is oestrogen intervention too late?

Fighting overwhelming cancer cell flexibility

The enemy is us, Haddow⁴⁸ in the Inaugural Karnofsky Lecture reasoned that it would not be possible to develop a cancer specific therapy in the same way Ehrlich had for syphilis, as cancer was our own cells. What he did not know was that the situation is worse than that. The replicative fidelity of normal cells replaces exactly what is lost, but in its own special place. Cytotoxic chemotherapy kills the patient by indiscriminately killing normal differentiated cells, and perhaps stem cells, so life saving repopulation for the host

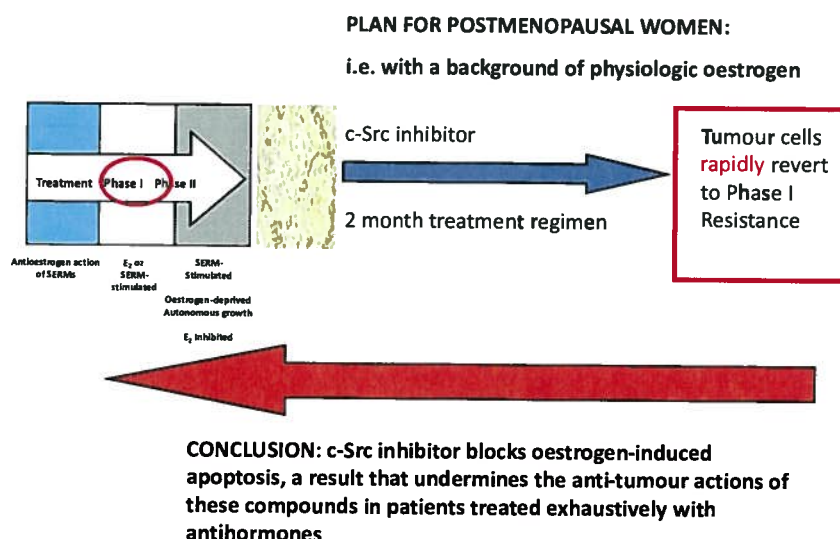


Fig. 10. The evolution of drug resistance and rapid alterations in cell populations if a c-Src inhibitor PP2 (5 μ M) is incubated with MCF-7:5C cells in the presence of 1nM oestradiol for two months to mimic a clinical scenario of a postmenopausal woman who fails an aromatase inhibitor to block growth. Apoptosis from oestrogen is blocked and the cells revert to Phase I resistance, i.e. oestrogen and SERM-stimulated growth.

organism is impossible or too late. In contrast, human populations eventually adapt to external destructive forces such as fatal infectious diseases (plague, small pox, etc.) but individuals only survive through their preprogrammed nimble immunology. The survivors repopulate. And so it is with cancer at the cellular level within the body. However, immunology has not yet been proven to be of significance for breast cancer prevention. Haddow was right there – the enemy is us. The tumour at diagnosis has hundreds of mutations compared to the (purportedly) normal human genome.^{65,66} This and activated oncogenes, or loss of tumour suppression genes, provides the random survival flexibility within the cancer cell population to adapt and eventually thrive in a hostile (cytotoxic) environment within a few months. The principle is a microscopic adaptation of simple Darwinian evolution that has played out over the millennia by animals on earth. Random mutations create a preferred trait that permits survival, while the non-adaptive species or population dies out. The situation with cancer only becomes worse through adaptive survival responses preprogrammed in the cancer stem cell. These cellular "spores" seek to expand and prepare for massive repopulation in an enforced anoxic environment. The clinician is confronted with a perverted microcosm of the struggle for life by cancer cells programmed to create infinite candidates in the quest for survival. The patient is overwhelmed by sheer numbers in the wrong places. This is the challenge of targeted molecular therapeutics but how to build rationally on the advances in survivorship achieved over the past 40-years in breast cancer?

The path to progress in drug development has not changed significantly during this time, despite our new knowledge of the disease. The administrative plan for drug evaluation is in place to protect citizens and provide safe and therapeutically proven medicines for clinical care. To market a new drug to treat breast cancer, a precise system must be followed to obtain government approval. Phase I clinical trials must offer the hope of potentially effective treatment to patients who have received all possible therapeutic options. The goal is to document dose limiting toxicities and at this stage of the disease, responses are a major bonus. Phase II trials focus on a cancer type of interest based on reasonable data from preclinical studies or an unanticipated response in Phase I trials. If a candidate is successful in Phase II trials, the drug is evaluated against or with the current standard of care. It should be emphasized that therapeutic results from Phase II trials with tamoxifen were not very dramatic, but Phase I data on toxicity for

the patient was excellent compared with other therapies available. Only by targeting the ER in the tumour and applying long-term adjuvant therapy did patient survivorship increase. A discarded contraceptive became the "gold standard" for breast cancer therapy over a 30-year journey.¹

With this background, how do we build on success? Today there are dozens of good potential targets and dozens of plausible candidates for each target. However, unlike the ER which was, it has turned out, the principle messenger to stimulate breast tumour growth in about 30% of tumours, other candidate targets are proving to be not the star but part of the chorus. In late stage disease, one pathway is blocked but others now compensate. Pathways to preserve cellular life can be essential in all cells, but a cancer cell specific pathway is the only key to success in cancer therapeutics.

Based on our current work investigating oestradiol-induced apoptosis of breast cancer cells with long-term acquired resistance, we proposed a hypothesis: can we block breast cancer cell survival mechanisms and enhance the chances that the cell must undergo apoptosis in response to oestradiol?

c-Src was the first identified oncogene in cancer and is said to be present in more than 70% of breast cancers.⁶⁷ It controls AKT and MAPK phosphorylation cascades as the intermediary from growth factor receptor activation. It would appear to be an ideal target to subvert cell survival; almost as good as the ER! We posed the question, that if we blocked c-Src in breast cancer cells resistant to aromatase inhibitors would we then enhance apoptosis? In other words, would we generate value for the cancer patient by increasing cell kill as we have previously found that c-Src inhibitors were completely ineffective in affecting growth of oestrogen stimulated MCF-7 cells, but had significant efficiency in blocking the growth of ER-negative MDA-MB-231 and oestrogen stimulated ER-positive T47D cells. More importantly, long-term oestrogen-deprived MCF-7 cells have elevated pSrc. As most ER-positive cancers are exhaustively treated with anti-hormones before Phase I/II testing and we were building on a known efficacy of estrogen therapy, the proposition appeared sound. Our model cell, MCF-7:5C, had elevated phospho c-Src and are targeted inhibitor PP2 completely blocked phosphorylation. However, a 2 month course of treatment of MCF-7:5C cells with physiological oestrogen levels (1nM) that would be present in a postmenopausal patient plus the c-Src inhibitor (5 microMolar), resulted in the blockade of oestrogen-induced apoptosis and the reversion of the cell population to

Phase I drug resistance (Fig. 10), i.e. estrogen or SERM-stimulated for growth. Within 2 months, the flexibility of cell populations had created no real advance that could realistically aid the patient.

Thus, as an illustration of the challenge we face for the application of logical targeted therapy, one could conclude the following: an expanding menu of targeted medicines is available for testing, but only select populations will respond. Testing a c-Src inhibitor in the incorrect stage of antihormone resistance or patient populations cannot be successful. This is the problem: the testing populations for registration may be inappropriate for a drug candidate that is magnificent in a neoadjuvant therapy naïve disease study. However, does this enhance registration? Unfortunately not.

We need practical strategies to aid communities to hold the development and death from breast cancer while we attempt to decipher the enormous complexity of pathways and permutations of targeted therapies. This conclusion brings us back to the second piece of translational research started in our laboratory in the 1970s – chemoprevention. Remarkably, the lamp of tamoxifen shed light on an alternative strategy to reduce cancer incidence and preempt the aforementioned Gordian Knot. Unfortunately, the initial strategy for the clinical application of chemoprevention requires the identification of high-risk populations to be treated with the pioneer tamoxifen. This approach is flawed. However, a public health strategy for an aging population that creates wellness for as long as possible is a laudable goal now within our grasp.

Tamoxifen is also about progress in chemoprevention

An extensive study of the pharmacology of tamoxifen⁶⁸ identified its ability to modulate oestrogen target tissues around the body; tamoxifen is anti-oestrogenic in the breast and oestrogen-like in bone and lowers circulating cholesterol.^{69–72} Translational research also first identified the potential of tamoxifen to increase the risk of developing endometrial cancer during extended treatment schedules.^{73–75} Tamoxifen blocks breast tumour growth and development but enhances endometrial cancer growth. As a result, new procedures were introduced for the gynecological monitoring of post-menopausal patients receiving long-term tamoxifen therapy. New agents, without endometrial problems, were needed for investigation. Knowledge of selective ER modulation by tamoxifen and also the pharmacology of the structurally-related failed breast cancer drug, raloxifene, led to the creation of a new drug group, the Selective ER Modulators (SERMs),⁷⁶ with the potential to treat and prevent multiple diseases in women **and** prevent breast cancer at the same time. The fact that raloxifene was less oestrogen-like than tamoxifen in the rodent uterus and less likely to increase the incidence of the endometrial cancer in patients^{77,78} meant that safer compounds could be identified as chemopreventives for breast cancer but a new strategy to achieve the goal was essential. Benefits for a tiny, unidentifiable minority is unacceptable if the vast majority of women in a high risk population have side effects, some life-threatening. The road map for the pharmaceutical industry was clearly stated in 1990. *Is this the end of the possible applications for anti-oestrogens? Certainly not! We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.*⁷⁹

Raloxifene pioneered the concept in the clinic confirming the prediction that the prevention of breast cancer would occur during the treatment and prevention of osteoporosis in high risk post-menopausal women⁸⁰ with no increase in endometrial cancer. Today, the prediction that SERMs could control multiple diseases in women following the menopause is poised to become a reality. Lasofoxifene (Fig. 1) is approved in the European Union for the prevention and treatment of osteoporosis which simultaneously decreases the incidence of breast cancer, strokes and myocardial infarction, but without increasing endometrial cancer risk.⁸¹ Lasofoxifene is more than one hundred times more potent than raloxifene and the aforementioned strategy⁷⁹ to improve women's health in aging populations is the new face of chemoprevention in breast cancer – treat the majority of women for major diseases like osteoporosis and coronary heart disease and prevent breast cancer as a beneficial side effect. The saving in health care costs by **not** paying for the treatment of breast cancer in tens of thousands of women **without** breast cancer will be considerable, but admittedly hard to quantitate.

Raloxifene is not only available in the United States of America for the treatment and prevention of osteoporosis but also for reduction of the incidence for breast cancer in post-menopausal, high-risk women.⁸² However, the SERM must be given indefinitely to remain effective in both diseases.⁸³ In contrast, tamoxifen remains effective for decades after the limited treatment period of 5 years is stopped.^{84,85} As mentioned previously, the key to understanding this fact probably resides in the laboratory study of drug resistance to SERMs and aromatase inhibitors and the development of a cellular susceptibility to oestrogen-induced apoptosis. The fact that the same tumour responsiveness to raloxifene appears to be retarded in clinical practice suggests that the known poor pharmacokinetics and bioavailability of raloxifene is not able to rapidly produce an "anti-oestrogenic" state around the nascent tumour like tamoxifen. This may explain the reduced performance of raloxifene against tamoxifen in the STAR trial, following the cessation of 5-years of treatment.⁸³

Summary and closing thoughts

Over the past 40 years, we have witnessed a dramatic improvement in the survivorship of the majority of patients with a diagnosis of ER positive breast cancer. The SERM tamoxifen pioneered the process. Translational research has added further cheap and effective targeted anti-hormonal therapies to the physician's armamentarium that are proven to be of benefit in randomized adjuvant clinical trials world-wide. Not only has therapy been improved substantially over the past 40 years, from the time in the early 1970s when there was stated to be little prospect of successful survival advances with "endocrine therapy", but also the parallel path of chemoprevention has been pioneered successfully with the same SERM tamoxifen. This SERM heralded a new era of general medicine where a family of SERMs would allow women to expect to reduce their risk of fractures but prevent breast cancer at the same time. This was only a laboratory concept 20-years ago^{79,86} but it seems obvious that with an aging population that seeks to remain active for as long as possible, that the SERMs will play their part in reducing the incidence of breast cancer if used wisely in the post-menopausal population.

The lessons learned with the lamp-light of the pioneer tamoxifen are now established principles in cancer therapeutics. The principles are: aim at the target (ER), start therapy as early as possible (i.e. as few lymph nodes as possible involved), long therapy is preferable to shorter therapy, compliance with the medicine is essential and drug interaction with SSRIs to stop menopausal side effect in a few should be avoided. Conforming to these principles aids patients' survival. The light from the lamp also taught us

what we did not know. Firstly tamoxifen is a selective modulator of ER action around a woman's body and heralded a new drug group (the SERMs) that prevent osteoporosis and prevent breast cancer as a beneficial side effect. This avoids the need to find the exact women who would benefit from chemoprevention using the Gail Model.⁸⁷ Secondly, drug resistance evolves so that oestradiol becomes an apoptotic trigger. Further studies solved the concern expressed by Haddow in 1970 that the mechanism of oestrogen-induced apoptosis was a mystery. Now oestrogen therapy has a niche application in patient care.

During the past 40 years, the mosaic of endocrine adjuvant therapy and chemoprevention with SERMs has been clarified by effective translational research.^{4,88} The next challenge for a generation of "omic" scientists is to prioritize the opportunities in molecular therapeutics based on this solid start, so as to advance and individualize treatment.

Acknowledgements

We would like to thank Julia Jessup Tijerina and Russell E. McDaniel for their administrative support in preparing this manuscript.

Conflict of interest statement

The authors have no conflict of interest to declare.

Funding

This work (VCJ) was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The project described was supported by Award Number P30 CA051008 from the National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

References

- Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;2:205–13.
- Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;44:30–8.
- Jordan VC. Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 2001;61:5683–7.
- Jordan VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death? *J Clin Oncol* 2008;26:3073–82.
- EBCTCG. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351:1451–67.
- EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687–717.
- Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 85 oestrogen receptor. *Eur J Cancer* 1975;11:205–6.
- Jordan V, Dix CJ, Allen KE. The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Ther Cancer* 1979;2:19–26.
- Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antiestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;16:239–51.
- Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antiestrogenic activity. *J Endocrinol* 1977;75:305–16.
- Jordan VC, Dix CJ, Naylor KE, Prestwich G, Rowsby L. Nonsteroidal antiestrogens: their biological effects and potential mechanisms of action. *J Toxicol Environ Health* 1978;4:363–90.
- Allen KE, Clark ER, Jordan VC. Evidence for the metabolic activation of non-steroidal antiestrogens: a study of structure-activity relationships. *Br J Pharmacol* 1980;71:83–91.
- NATO. Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1983;1:257–61.
- NATO. Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. Analysis at six years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1985;1:836–40.
- Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. *Br J Cancer* 1971;25:270–5.
- Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;72:7–25.
- Howell A, Cuzick J, Baum M, Buzdar A, Dowsett M, Forbes JF, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 2005;365:60–2.
- Hershman DL, Kushi LH, Shao T, Buono D, Kershenbaum A, Tsai WY, et al. Early discontinuation and nonadherence to adjuvant hormonal therapy in a cohort of 8,769 early-stage breast cancer patients. *J Clin Oncol* 2010;28:4120–8.
- Partridge AH, LaFountain A, Mayer E, Taylor BS, Winer E, Anis-Aliibozek A. Adherence to initial adjuvant anastrozole therapy among women with early-stage breast cancer. *J Clin Oncol* 2008;26:556–62.
- Lash TL, Fox MP, Westrup JL, Fink AK, Silliman RA. Adherence to tamoxifen over the five-year course. *Breast Cancer Res Treat* 2006;99:215–20.
- McCowan C, Shearer J, Donnan PT, Dewar JA, Crilly M, Thompson AM, et al. Cohort study examining tamoxifen adherence and its relationship to mortality in women with breast cancer. *Br J Cancer* 2008;99:1763–8.
- Geiger AM, Thwin SS, Lash TL, Buist DS, Prout MN, Wei F, et al. Recurrences and second primary breast cancers in older women with initial early-stage disease. *Cancer* 2007;109:966–74.
- Yood MU, Owusu C, Buist DS, Geiger AM, Field TS, Thwin SS, et al. Mortality impact of less-than-standard therapy in older breast cancer patients. *J Am Coll Surg* 2008;206:66–75.
- Brauch H, Jordan VC. Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the 'personalised' approach? *Eur J Cancer* 2009;45:2274–83.
- Dezentje VO, van Blijderveld NJ, Gelderblom H, Putter H, van Herk-Sukel MP, Casparie MK, et al. Effect of concomitant CYP2D6 inhibitor use and tamoxifen adherence on breast cancer recurrence in early-stage breast cancer. *J Clin Oncol* 2010;28:2423–9.
- Lash TL, Cronin-Fenton D, Ahern TP, Rosenberg CL, Lunetta KL, Silliman RA, et al. Breast cancer recurrence risk related to concurrent use of SSRI antidepressants and tamoxifen. *Acta Oncol* 2010;49:305–12.
- Kelly CM, Juurlink DN, Gomes T, Duong-Hua M, Pritchard KI, Austin PC, et al. Selective serotonin reuptake inhibitors and breast cancer mortality in women receiving tamoxifen: a population based cohort study. *BMJ* 2010;340:c693.
- SCOT. Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee, Scottish Cancer Trials Office (MRC), Edinburgh. *Lancet* 1987;2:171–5.
- Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res* 1997;57:3071–8.
- Osborne CK, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987;23:1189–96.
- Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207–13.
- Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;48:5183–7.
- Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* 1989;49:4090–3.
- Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991;51:3867–73.
- Osborne CK, Phippen J, Jones SE, Parker LM, Ellis M, Come S, et al. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* 2002;20:3386–95.
- Howell A, Robertson JF, Quaresima Albano J, Aschermannova A, Mauriac L, Kleeberg UR, et al. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002;20:3396–403.
- Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 1993;127:23–33.
- Yao K, Lee ES, Bentrem DJ, England G, Schafer JL, O'Regan RM, et al. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028–36.
- Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 2001;93:1714–23.

40. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, et al. Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;**95**:1586–97.
41. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;**95**:1597–608.
42. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;**97**:1746–59.
43. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, et al. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;**67**:111–6.
44. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 2009;**302**:774–80.
45. Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, et al. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 2010;**37**:387–98.
46. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;**94**:131–41.
47. Haddow A, Watkinson JM, Paterson E, Koller PC. Influence of synthetic oestrogens on advanced malignant disease. *Br Med J* 1944;**2**:393–8.
48. Haddow A. David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 1970;**26**:737–54.
49. Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, et al. Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 2008;**10**:R104.
50. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 1998;**95**:927–37.
51. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;**389**:753–8.
52. Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 1998;**58**:1872–5.
53. MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* 2000;**60**:5097–105.
54. Liu H, Lee ES, Deb Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor modulator–estrogen receptor alpha complex. *Cancer Res* 2001;**61**:3632–9.
55. Liu H, Park WC, Bentrem DJ, McKian KP, Reyes Ade L, Loweth JA, et al. Structure–function relationships of the raloxifene–estrogen receptor–alpha complex for regulating transforming growth factor–alpha expression in breast cancer cells. *J Biol Chem* 2002;**277**:9189–98.
56. Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, et al. Molecular classification of estrogens. *Cancer Res* 2001;**61**:6619–23.
57. Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, Jordan VC. Structure–function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 2010;**53**:3273–83.
58. Sengupta S, Sharma CG, Jordan VC. Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Horm Mol Biol Clin Investig* 2010;**2**:235–43.
59. Bourgoin-Voillard S, Gallo D, Laios I, Cleeren A, Bali LE, Jacquot Y, et al. Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif – Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* 2010;**79**:746–57.
60. Lonning PE. Additive endocrine therapy for advanced breast cancer – back to the future. *Acta Oncol* 2009;**48**:1092–101.
61. Stefanick ML, Anderson GL, Margolis KL, Hendrix SL, Rodabough RJ, Paskett ED, et al. Effects of conjugated equine estrogens on breast cancer and mammography screening in postmenopausal women with hysterectomy. *JAMA* 2006;**295**:1647–57.
62. Beral V, Reeves G, Bull D, Green J. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. *J Natl Cancer Inst* 2011;**103**:296–305.
63. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 2009;**11**:206.
64. Maximov PY, Lewis-Wambi JS, Jordan VC. The paradox of oestradiol-induced breast cancer cell growth and apoptosis. *Curr Signal Transduct Ther* 2009;**4**:88–102.
65. Berger MF, Lawrence MS, Demicheli F, Drier Y, Cibulskis K, Sivachenko AY, et al. The genomic complexity of primary human prostate cancer. *Nature* 2011;**470**:214–20.
66. Pfeifer GP, Hainaut P. Next-generation sequencing: emerging lessons on the origins of human cancer. *Curr Opin Oncol* 2011;**23**:62–8.
67. Ottenhoff-Kalff AE, Rijkse G, van Beurden EA, Hennipman A, Michels AA, Staal GE. Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-Src oncogene product. *Cancer Res* 1992;**52**:4773–8.
68. Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 2006;**147**(Suppl 1):S269–76.
69. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;**46**:1870–4.
70. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;**10**:31–5.
71. Love RR, Mazess RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;**326**:852–6.
72. Love RR, Wiebe DA, Newcomb PA, Cameron L, Leventhal H, Jordan VC, et al. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med* 1991;**115**:860–4.
73. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;**48**:812–5.
74. Fornander T, Cedermark B, Mattsson A, Skoog L, Theve T, Askergren J, et al. Adjuvant tamoxifen in early breast-cancer – occurrence of new primary cancers. *Lancet* 1989;**1**:117–20.
75. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;**90**:1371–88.
76. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 2004;**25**:45–71.
77. Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothienophene derived antiestrogen. *Life Sci* 1983;**32**:1031–6.
78. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res* 1990;**50**:3189–92.
79. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 1990;**50**:4177–89.
80. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 1999;**281**:2189–97.
81. Cummings SR, Ensrud K, Delmas PD, LaCroix AZ, Vukicevic S, Reid DM, et al. Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med* 2010;**362**:686–96.
82. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 2006;**295**:2727–41.
83. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 2010;**3**:696–706.
84. Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;**97**:1652–62.
85. Powles TJ, Ashley S, Tidy A, Smith IE, Dowsett M. Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst* 2007;**99**:283–90.
86. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest* 1988;**6**:589–95.
87. Gail MH, Brinton LA, Byar DP, Corle DK, Green SB, Schairer C, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst* 1989;**81**:1879–86.
88. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 2009;**69**:1243–54.

Proteomic Analysis of Pathways Involved in Estrogen-Induced Growth and Apoptosis of Breast Cancer Cells

Zhang-Zhi Hu^{1,2*}, Benjamin L. Kagan^{1*}, Eric A. Ariazi³, Dean S. Rosenthal¹, Lihua Zhang¹, Jordan V. Li¹, Hongzhan Huang², Cathy Wu², V. Craig Jordan¹, Anna T. Riegel¹, Anton Wellstein^{1*}

1 Lombardi Cancer Center, Georgetown University, Washington, D.C., United States of America, **2** Protein Information Resource, Georgetown University, Washington, D.C., United States of America, **3** Fox Chase Cancer Center, Philadelphia, Pennsylvania, United States of America

Abstract

Background: Estrogen is a known growth promoter for estrogen receptor (ER)-positive breast cancer cells. Paradoxically, in breast cancer cells that have been chronically deprived of estrogen stimulation, re-introduction of the hormone can induce apoptosis.

Methodology/Principal Findings: Here, we sought to identify signaling networks that are triggered by estradiol (E2) in isogenic MCF-7 breast cancer cells that undergo apoptosis (MCF-7:5C) versus cells that proliferate upon exposure to E2 (MCF-7). The nuclear receptor co-activator AIB1 (Amplified in Breast Cancer-1) is known to be rate-limiting for E2-induced cell survival responses in MCF-7 cells and was found here to also be required for the induction of apoptosis by E2 in the MCF-7:5C cells. Proteins that interact with AIB1 as well as complexes that contain tyrosine phosphorylated proteins were isolated by immunoprecipitation and identified by mass spectrometry (MS) at baseline and after a brief exposure to E2 for two hours. Bioinformatic network analyses of the identified protein interactions were then used to analyze E2 signaling pathways that trigger apoptosis versus survival. Comparison of MS data with a computationally-predicted AIB1 interaction network showed that 26 proteins identified in this study are within this network, and are involved in signal transduction, transcription, cell cycle regulation and protein degradation.

Conclusions: G-protein-coupled receptors, PI3 kinase, Wnt and Notch signaling pathways were most strongly associated with E2-induced proliferation or apoptosis and are integrated here into a global AIB1 signaling network that controls qualitatively distinct responses to estrogen.

Citation: Hu Z-Z, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, et al. (2011) Proteomic Analysis of Pathways Involved in Estrogen-Induced Growth and Apoptosis of Breast Cancer Cells. PLoS ONE 6(6): e20410. doi:10.1371/journal.pone.0020410

Editor: Rakesh K. Srivastava, The University of Kansas Medical Center, United States of America

Received: February 7, 2011; **Accepted:** April 23, 2011; **Published:** June 27, 2011

Copyright: © 2011 Hu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Department of Defense Breast Program W81XWH-06-10590 Center of Excellence Grant (V.C.J., A.W., A.T.R.), National Institutes of Health/National Cancer Institute R01 CA113477 (A.T.R.) and P30 CA051008. Views and opinions of, and endorsements by the author(s) do not reflect those of the U.S. Army or the Department of Defense. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wellstea@georgetown.edu

These authors contributed equally to this work.

Introduction

Estrogen induces proliferation of estrogen receptor (ER)-positive breast cancer cells [1]. This response is consistent with the finding that antihormone therapies, such as tamoxifen or aromatase inhibitors, can enhance survivorship and reduce recurrence in patients with ER-positive breast cancers [2,3]. However, the majority of tumors eventually become unresponsive to antihormone treatments [4,5] and molecular mechanisms and markers of antihormone resistance have been described [6,7]. Once patients have failed on antihormone therapy, one treatment option has been the use of pharmacologic doses of estrogens [8,9] based on well-established findings that some breast cancers shrink during high dose estrogen treatment [10,11,12]. This phenomenon has also been observed in laboratory models of ER-positive breast cancer with acquired anti-hormone resistance that regress and undergo apoptosis in the presence of physiologic concentrations of estrogen [13,14] and was reviewed recently for its potential clinical implications [15].

Estrogen exerts diverse effects including genomic and non-genomic effects through multiple signaling pathways, that are significantly altered in anti-hormone resistant ER positive breast cancer cells. In antihormone resistant cells, for example, there is a general increase in EGFR and IGFR tyrosine kinase signaling [16,17], accompanied by increased ligand-independent phosphorylation of ER [18] and nuclear receptor co-activators such as AIB1/SRC3 (Amplified in Breast Cancer 1/Steroid Receptor Co-activator3) [19]. Overexpression and activation of AIB1 is associated with endocrine resistance in human breast cancer [20,21,22] and has been shown to be rate-limiting for estrogen-induced growth of breast cancer cells [23,24]. Beyond its role in these effects of estrogen, AIB1 was also shown to be rate-limiting for the growth of estrogen-insensitive breast cancer cells [25] as well as prostate cancer [26], pancreatic cancer [27] and lymphoma cells [28]. Furthermore, in AIB1 knockout mice, responses to hormones [29] as well as growth factor signaling [30] are blunted whereas overexpression of an AIB1 transgene leads to increased estrogen and growth factor responses resulting in

hyperplasia and neoplasia of mammary glands [31,32,33]. Thus, a large body of data support a crucial role for AIB1 in estrogen and growth factor signaling (reviewed in Refs [34,35]) and provides the rationale for the experimental paradigm used here.

To identify pathways that initiate estrogen-induced apoptosis versus growth, we used a combined proteomics and systems biology approach to elucidate triggering events and associated signaling pathways. We focused on changes of AIB1 interacting proteins, because of its central role in estrogen control of phenotypic behavior of breast cancer cells outlined above. AIB1 also coactivates IGF1R, EGFR and HER2 through modulation of tyrosine phosphorylation of these transmembrane receptors and phosphorylation of their subsequent signaling intermediaries [27,30,33,34]. Thus, to complement the analysis of direct AIB1 interacting proteins, we also monitored changes of phosphotyrosine (pY)-containing protein complexes, that are most likely regulated by growth factor signaling, as a means of discovering global intersecting pathways. As a model system, we used MCF-7 cells that proliferate in response to E2 [1], but also respond to EGF and heregulin [36] and have high levels of AIB1 protein due to gene amplification [37]. Wild-type MCF-7 cells were compared with MCF-7:5C cells that had been isolated under estrogen-free

growth conditions [38,39]. MCF-7:5C cells were derived following long-term culture of MCF-7 cells in phenol red-free media. MCF-7:5C cells are ER-positive and undergo apoptosis after exposure to physiological concentrations of E2. In contrast, wild-type parental MCF-7 cells proliferate in the presence of the same concentration range of E2 [38,39]. The MCF-7:5C cells represent many of the characteristics of Phase II SERM resistant cells [40]. A parallel analysis after estrogen stimulation of these isogenic breast cancer cell lines served as a basis for the comparisons of signaling responses.

Here, we show that RNAi-mediated depletion of AIB1 reduces E2-induced growth of MCF-7 cells, and reverses the estrogen-induced apoptosis in MCF-7:5C cells. AIB1-interacting and pY-containing protein complexes were immunoprecipitated from short-term E2-treated cells, and the complexed proteins were identified by mass spectrometry (MS) analysis (Fig. 1A). From a comparison of the data sets obtained with MCF-7 versus MCF-7:5C cells treated with or without E2, and from a computationally-derived global AIB1-interacting network prediction, we identified pathways that participate in the differential response to E2 in these breast cancer cells. We found that a limited number of major cellular signaling pathways i.e. GPCR, PI3 kinase, Wnt, Notch

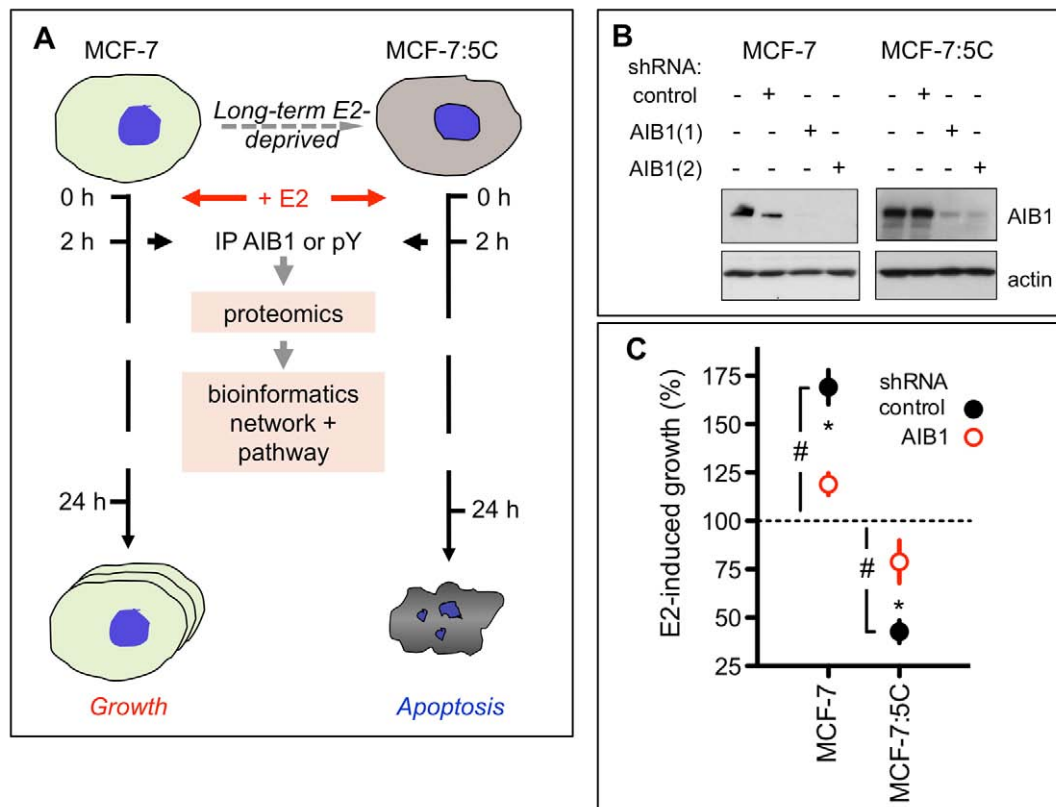


Figure 1. Phenotypic impact of AIB1 depletion on estradiol (E2) growth response in MCF-7 or MCF-7:5C cells. (A) The experimental paradigm. The differential responses to estradiol (E2) treatment of MCF-7 (cell growth) and long-term estrogen deprived MCF-7:5C cells (apoptosis) are indicated. Proteomics profiles of the two cell lines at baseline and after a brief (2 h) E2 treatment were generated using immunoprecipitations (IP). Proteins interacting with AIB1 or phosphotyrosine containing protein complexes were isolated by IP followed by mass spectrometry. Data were then subjected to an integrated bioinformatics analysis of signaling pathways and protein networks. (B,C) Reversal of E2-dependent effects on MCF-7 and MCF-7:5C after depletion of endogenous AIB1 protein using two different lentiviral shRNAs. MCF-7 or MCF-7:5C cells were infected with lentiviral particles expressing control or AIB1-targeting shRNAs. (B) RNAi-mediated knockdown was assayed by Western blot analysis for AIB1 relative to an actin loading control. (C) Cell growth was assayed 6 days after plating without or with E2. The E2 effect is shown relative to the respective untreated controls (mean \pm S.E.M.). Closed circles: control shRNA; Open circles (red): AIB1 shRNA. #, $p < 0.05$ E2 treatment effect vs. no treatment in control shRNA cells; *, $p < 0.05$ E2 treatment effect in control shRNA cells vs. E2 treatment in AIB1 depleted cells. Representative data from one of at least three independent experiments are shown. doi:10.1371/journal.pone.0020410.g001

and their associated molecules were involved in the control of estrogen induced proliferative or apoptotic responses. This information will be useful for determining appropriate targets to induce apoptosis in endocrine resistant human breast cancer.

Results and Discussion

Impact of AIB1 depletion on E2-induced growth effects in MCF-7 and MCF-7:5C cells

To determine the role of AIB1 in the E2-induced, distinct growth phenotypes of MCF-7:5C and wild-type MCF-7 cells, both cell lines were infected with lentiviral vectors that express control or two distinct AIB1-targeted shRNAs, and selected in puromycin for stable integrants. Both MCF-7 and MCF-7:5C cells were depleted of AIB1 protein, compared to uninfected and control shRNA infected cells with either of the shRNAs (Fig. 1B). Treatment with E2 significantly induced growth of control shRNA-infected MCF-7 cells and reduced the growth of MCF-7:5C cells (Fig. 1C, black symbols). In contrast to this, in AIB1-depleted, wild-type MCF-7 cells, E2 did not stimulate growth significantly above baseline and in AIB1 depleted MCF-7:5C, E2 lost its apoptosis-inducing effect (Fig. 1C, red symbols). These data suggest that AIB1 is a significant control hub of the E2-controlled growth phenotype in these ER-positive breast cancer cells.

Global analysis of AIB1- and phosphotyrosine-complexed proteins

Because AIB1 is rate-limiting for the E2-induced changes in the growth phenotype of MCF-7 and MCF-7:5C cells, we performed AIB1-specific immunoprecipitations of lysates from untreated and E2-treated (2 hrs) MCF-7 and MCF-7:5C cells to fractionate the respective proteome. Immunoprecipitation of phosphotyrosine-containing protein complexes was also performed to complement the AIB1-specific proteome fractionation (Fig. 1A). The immunoprecipitates were released from the beads, separated by denaturing gel electrophoreses (SDS-PAGE) and followed by Coomassie Blue staining of proteins in the gels (Fig. S7). Visible bands and the same region in parallel gel lanes were harvested and proteins present identified by mass spectrometry (MS). Stringent filtering of the initial proteomic data resulted in a subset of 101 proteins that either interacted with AIB1 ($n=58$, Table S1) or are present in pY-protein complexes ($n=56$, Table S2), with 13 proteins common to both.

The analytical approach emphasizes reliable identification of proteins by correlating mass spectrometry ID with the apparent molecular mass obtained from the SDS-PAGE (Fig. S7). This approach mimics Western blotting without having to rely on the availability of antibodies, appropriate sensitivity, suitability for Western blotting and specificity. Still, we used Western blotting of some proteins identified by MS and show two examples in Fig. S8 (see below). To validate the mass spectrometry findings, separate experiments with independent mass spectrometry analyses were run. We found 48% of the proteins reported here in two and 16% in three or more independent experiments. This compares favorably with a recent HUPO study where only 7 of 27 laboratories identified all 20 proteins present at equimolar concentrations in a test sample [41]. In our experiments, the abundance of individual endogenous proteins captured in the immunoprecipitates covers a wide range (see Fig. S7). Thus, we expected that lower abundance proteins may drop below detection in repeat experiments. A combination of bioinformatics and mass spectrometry analysis was thus applied to meet this challenge as also described elsewhere [42,43].

The Venn diagrams of proteins pulled down with anti-AIB1 or anti-pY (Fig. 2) show the distribution of proteins between E2-treated and untreated, as well as wild-type MCF-7 versus MCF-7:5C cells (A and B), or between E2-treated and untreated cells regardless of cell type (C, *top*; and D, *top*), or between MCF-7 and MCF-7:5C cells regardless of treatment (C, *bottom*; and D, *bottom*). The number of pY-complexed proteins identified was affected very little by E2 treatment (18 vs. 25 proteins) with 13 proteins in either treatment group (Fig. 2D). In contrast, there was a significant, 4-fold higher number of AIB1-interacting proteins in the E2-treatment group (8 vs. 33 proteins; $p<0.05$, chi-square test; Fig. 2C) with 17 proteins not impacted in their interaction with AIB1. This suggests that AIB1-mediated protein-protein interactions are more responsive to E2 treatment, and new protein complexes are induced by E2 (Fig. 2A,C). In addition, the total number of proteins in complexes with AIB1 that overlap between MCF-7 and MCF-7:5C cells was not altered by the treatment, although the fraction of proteins per cell line that overlap decreases by 1/2 with E2-treatment (31% to 16%; Fig. 2A). Finally, while pathways activated by E2 gave rise to different sets of pY-containing protein complexes in both MCF-7 and MCF-7:5C cells, the percentage of proteins that overlap between cell lines remain almost constant regardless of treatment (4 vs. 5 in Fig. 2B).

Figure 3 shows the functional categories ascribed to the AIB1-associated (top) and pY-complexed (bottom) proteins. Tables S1 and S2 identify the proteins in each of these categories, cell lines (MCF-7 versus MCF-7:5C), and conditions (+/− E2) under which they were identified. Nearly half of the AIB1-interacting proteins fall into four categories, i.e. cytoskeleton and structural proteins, metabolism, transcription regulation, and signal transduction. Most of the pY-complexed proteins fall into four major functional categories: cytoskeleton and structural proteins, transcription regulation, signal transduction, and protein transport and vesicle trafficking. Thirteen proteins were found to be both AIB1-interacting and pY-complexed in MCF-7 and MCF-7:5C cells (Table S1).

Distinct profiles were observed for metabolism-related proteins between AIB1- and pY-complexed proteins, where the AIB1 complexes contained eight different enzymes in contrast to only one in the anti-pY group. This is consistent with studies demonstrating that AIB1 plays a role in the control of basal metabolic processes [44,45] that resulted in growth retardation and reduced hormonal responses in AIB1 knock-out mice [46]. Quite strikingly, all of these proteins were identified in E2 treated cells (e.g. 5-oxoprolinase in MCF-7:5C and fatty acid synthase in MCF-7 cells), whereas only three were identified in untreated as well as E2 treated cells. Seven AIB1-interacting proteins were detected in the categories of transcriptional regulation and chromatin complex, consistent with the role of AIB1 as a transcriptional coactivator. Interestingly, several proteins were found with pY immunoprecipitation that were unique to E2-treated MCF-7:5C cells, one of which was FAK1 (PTK2; Table S2). FAK1 is known to complex with EGFR as well as with an isoform of AIB1 and thus contribute to cellular signaling in breast cancer cells [47]. The MS based identification of FAK1 in the anti-pY immunoprecipitates was also seen by Western blot (Fig. S8A).

AIB1-containing protein complexes in E2-treated MCF-7:5C cells

We identified 18 proteins (CI >95%) that interact with AIB1 in E2-treated but not in untreated MCF-7:5C cells, 10 of which are also unique to MCF-7:5C cells (Table S1; Fig. 2A). These E2-induced AIB1-interacting proteins in MCF-7:5C cells mainly segregate in the category “transcriptional regulation” (6 of 18),

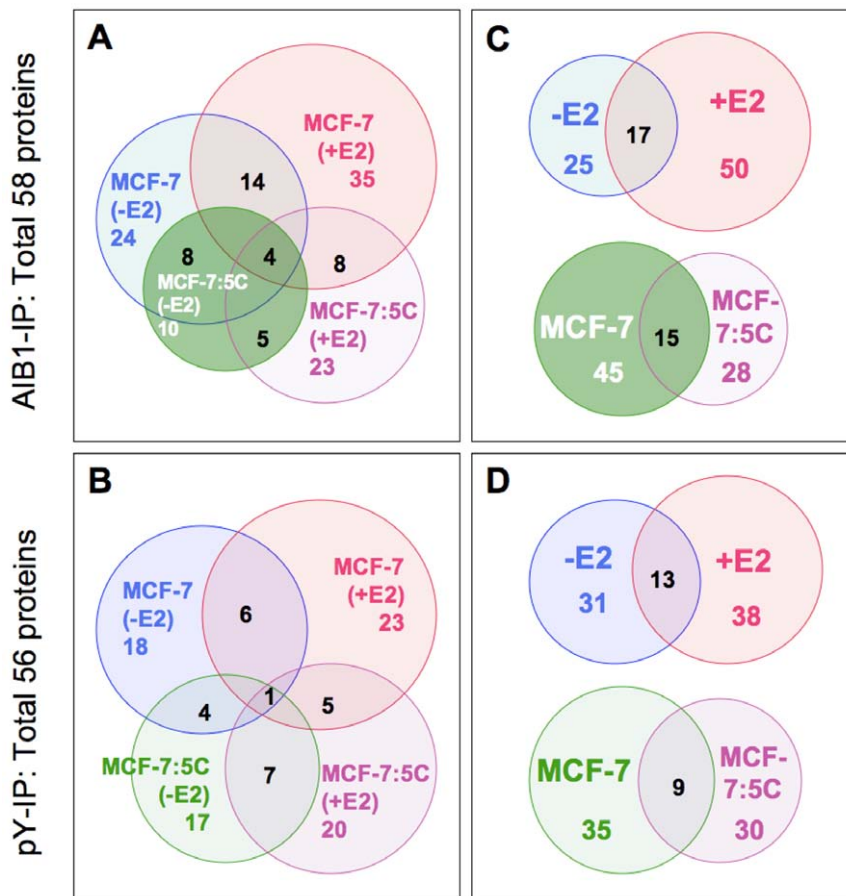


Figure 2. Summary of proteins identified under different conditions. Venn diagrams of proteins identified from anti-AIB1 (A,C) or anti-pY IP (B,D) experimental groups. (C,D) Proteins in combined AIB1-IP or pY-IP data sets. Individual proteins and subgroups are shown in Tables S1 & S2. doi:10.1371/journal.pone.0020410.g002

several of which are also known to be involved in the control of apoptosis. For example, PRDM5, a PR domain and zinc-finger transcriptional regulator is a putative tumor suppressor and has been linked to cancer cell apoptosis [48]. TLE3, a transcriptional corepressor that binds to a number of transcription factors [49], can form a transcriptional repressor complex with RUNX3 [50], a known tumor suppressor that has been shown to be involved in apoptosis in gastric and colon cancer [51]. TLE3 has also been associated with the development of anti-estrogen resistance [52]. The MS identification of the 83 kDa TLE3 in AIB1 immunoprecipitations (IP) by was also seen by Western blot analysis (Fig. S8B). IASPP was identified in complex with AIB1 in both E2-treated MCF-7 and MCF-7:5C cells, but not in untreated cells. IASPP, a member of ASPP family of proteins, exerts anti-apoptosis effects through modulation of p53 [53,54,55]. Interestingly PRPF6, identified here as AIB1-interacting, is an U5 snRNP-associated protein involved in pre-mRNA splicing and has been shown to be a coactivator of the androgen receptor and mediates its ligand-independent AF-1 activation [56]. TLE3, PRDM5 and PRPF6 were all uniquely identified in E2-treated MCF-7:5C cells.

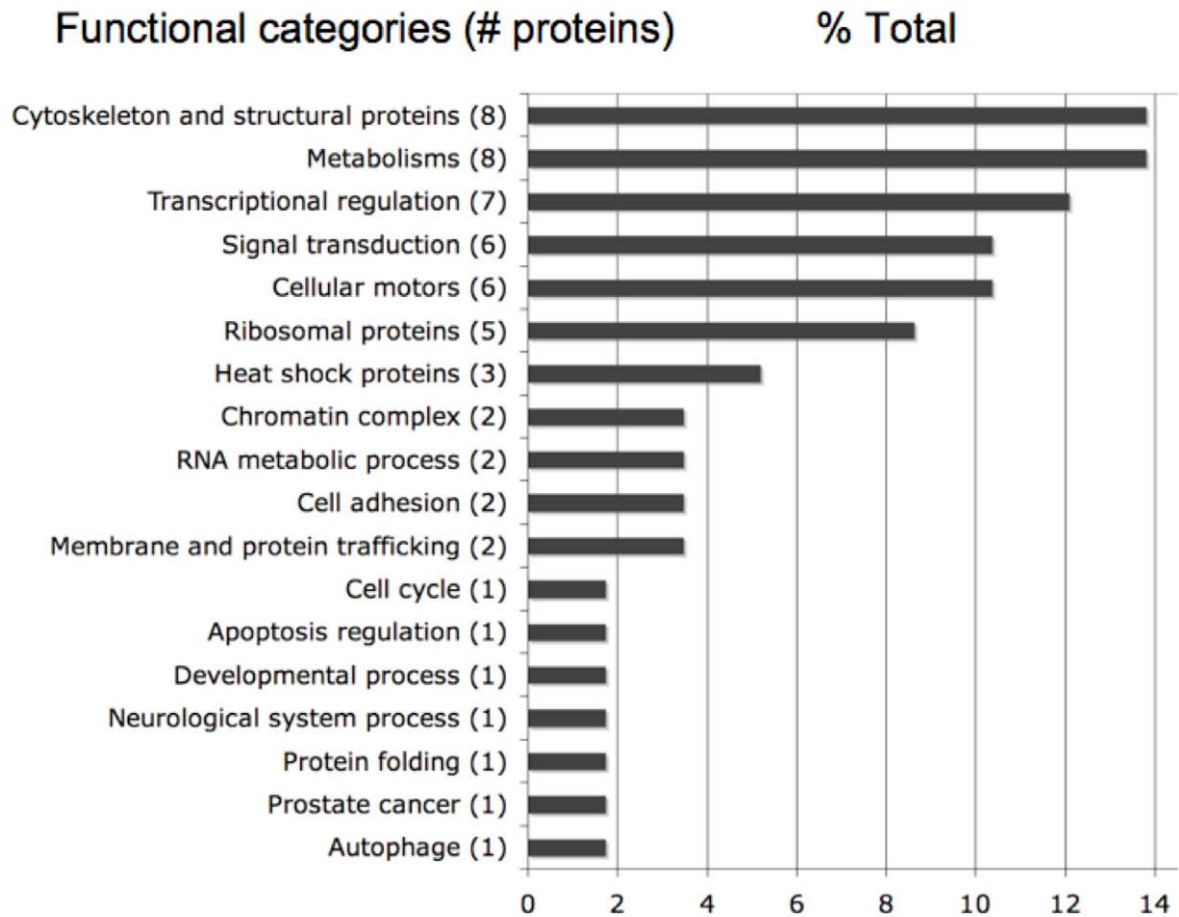
Potential pathways involved in E2-induced growth and apoptosis

To increase the potential of identifying pathways participating in E2-induced growth and apoptosis from the MS data sets, we not only analyzed proteins identified from MS with high confidence

(CI $\geq 95\%$), but also took a global approach to include all proteins identified at various CI levels (see <http://pir.georgetown.edu/iproxpress/coe2>) by MS before filtering for pathway mapping with the IngenuityTM and GeneGOTM pathway tools [43]. We hypothesized that if proteins identified at lower-level confidence by MS are found in known pathways that are consistent with the cellular phenotypes, they may provide valuable mechanistic insights. Also, supporting this approach are data from a recent study [57] with immunoprecipitation of nuclear extracts from MCF-7 cells that identified 13 of the 15 proteins we had seen at CI values in the lower range of 42–90%. The canonical pathway mapping analyses of all identified proteins suggest that several pathways are significantly represented both for proteins immunoprecipitated with anti-AIB1 and for those with anti-pY, including GPCRs, apoptosis, PI3K/AKT, and Wnt/ β -catenin and Notch signaling pathways (Fig. S1, S2, S3, S4):

GPCR and growth factor signaling. Figure S1 depicts the GPCR-induced cell growth pathway, in which a number of proteins were identified in both AIB1 and pY-associated complexes. G α (o) (GNAO2, IP-pY) and Rap1GAP (IP-AIB1) (Table S3), for example were identified exclusively in E2-treated MCF-7:5C cells. G α (o) has been shown to directly bind to Rap1GAP resulting in the inhibition of the Ras-MAPK proliferation pathway [58]. In E2-treated MCF-7 cells, G α (s) (GAS, GNAS) and CALM1 were coimmunoprecipitated with AIB1, while IP3R (ITPR3) was coimmunoprecipitated with AIB1 in both E2 treated MCF-7 and MCF-7:5C cells (Table S3). Each

AIB1-immunoprecipitated proteins (58)



pY-immunoprecipitated proteins (56)

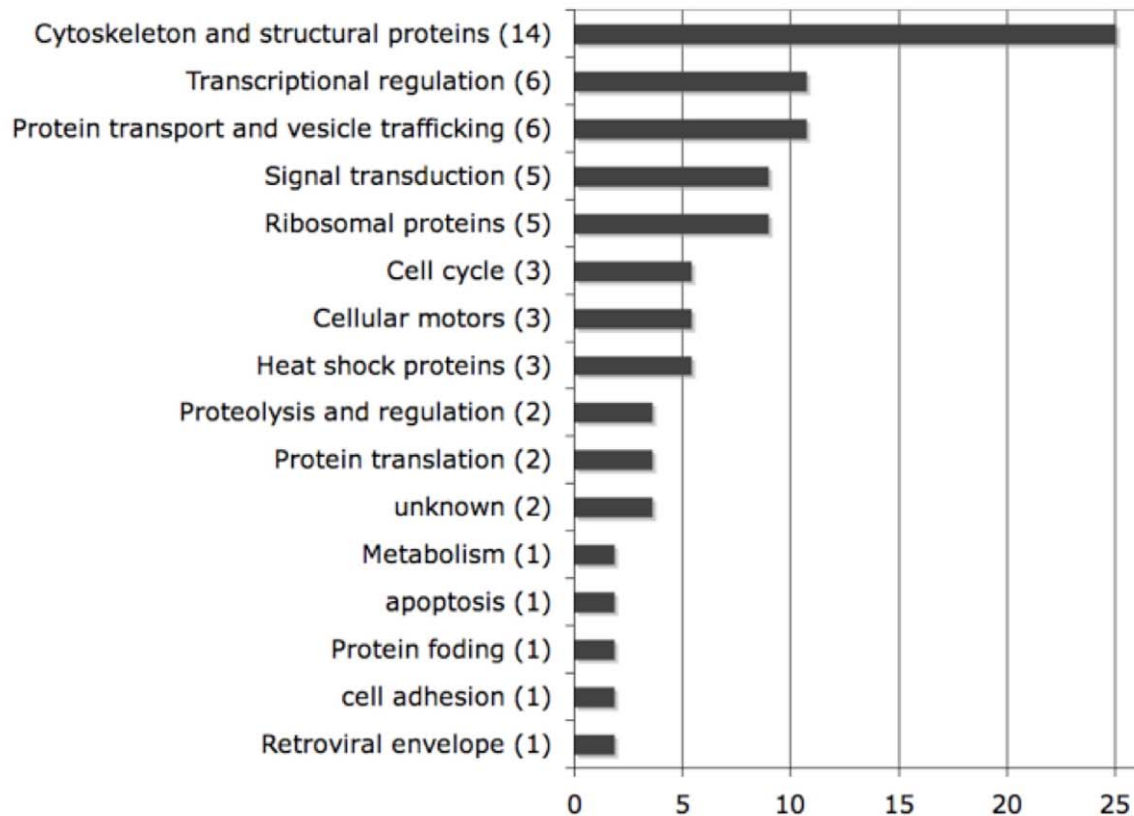


Figure 3. Functional categories of anti-AIB1 (upper) and anti-pY immunoprecipitated proteins (lower) from MCF-7 and MCF-7:5C breast cancer cells. Numbers in parenthesis are the number of proteins belonging to the respective category. Proteins profiled are those with CI values $\geq 95\%$ from mass spectrometry. doi:10.1371/journal.pone.0020410.g003

of these proteins is found downstream of GPCRs, and could lead to MAPK pathway activation and cell proliferation.

GPCRs and growth factors (IGF-1 and EGF) act via phosphorylation of the proapoptotic Bcl-2 family member BAD to regulate mitochondrial-mediated apoptosis (Fig. S2). BAD has been shown to be phosphorylated by Cdc2 (CDK1) at S128 [59] and Cdc2 was identified by anti-pY immunoprecipitation in E2-treated MCF-7:5C cells (Table S2). Also, two phosphatases, PP2B (PPP3CB) and PP2C (WIP1; Table S3, Fig. S2), associated with AIB1 only in MCF-7 cells. Both phosphatases can dephosphorylate BAD and thus modulate apoptosis [60]. In addition, RSK1 and RSK2, identified only in E2-treated cells (Table S3, Fig. S2), are also known to modulate cell survival [61,62].

Growth factors and cytokines can induce cellular growth and proliferation through PI3K-AKT signaling. A number of proteins complexed with AIB1 were identified in this pathway under different conditions (Fig. S3 and Table S3). The non-receptor tyrosine kinase TYK2 was detected in both MCF-7 and MCF-7:5C cells with or without E2 treatment. Both PI3K catalytic (p110) and regulatory (p85) subunits were pulled down only in E2-treated, not in untreated MCF-7 cells (Fig. S3C). PI3K/p110 was detected, additionally, in untreated but not treated MCF-7:5C cells (Fig. S3B). Thus, PI3K/p110 was isolated only under conditions that promoted proliferation in both cell lines. GSK3 β , identified in AIB1 immunoprecipitates in E2-treated MCF-7 cells (Fig. S3C), can be activated by PI3K/AKT, and has also been shown to be a regulator of Wnt signaling (see below). Finally, BCL3, a member of the I-kappa-B family that regulates NF κ B-mediated transcription [63,64], was only identified in E2-treated MCF-7 cells.

Wnt/ β -catenin and Notch signaling. Our data indicate that Wnt/ β -catenin, and Notch signaling pathways participate in E2 responses in both MCF-7 and MCF-7:5C cells (Fig. S4). Several key proteins in the pathway, such as Wnt ligands, cadherin, β -catenin, casein kinases and GSK3 β were identified in distinct AIB1- and pY-containing complexes, amongst different cells and treatments (Fig. S4A, B and C). For example, in MCF-7:5C cells, Frizzled-7 (FZD7) and cadherin 22 (CDH22) were identified in pY-containing complexes after E2 treatment, while β -catenin associated with AIB1 regardless of E2 treatment (Table S3). In MCF-7 cells, the Wnt ligand Wnt-7a, CK1 δ , and GSK3 β were identified in AIB1 immunoprecipitates (Table S3). CK1 δ was recently reported to modulate the transcriptional activity of ER α in an estrogen-dependent manner and regulates ER-AIB1 interactions [65]. An additional protein, δ -catenin, or p120^{cas}, a member of armadillo/ β -catenin superfamily [66], was identified in the AIB1 immunoprecipitates of E2-treated MCF-7 cells (Table S1).

Our results suggest that multiple proteins found in AIB1 associated complexes, that function in Wnt signaling, also crosstalk with Notch and growth factor-induced signaling in response to E2 treatment in breast cancer cells. TLE3 was detected only in E2-treated MCF-7:5C cells, and Notch1, Notch3, and Numb-like protein were identified only in E2-treated MCF-7 cells (Table S3). TLE3, the mammalian homolog of Gro [67], is a global corepressor mediating transcriptional repression targeted by a number of signal pathways. As shown in Fig. S4D, TLE3 connects the Notch and Wnt pathways [68,69]. In addition to the apoptosis related proteins discussed above (TLE3, PRDM5, CDK1), DBC1 was isolated from anti-pY immunoprecipitates in E2 treated MCF-

7:5C cells (Table S2). Interestingly, DBC1 was recently reported to increase p53 mediated apoptosis in breast cancer cells [70]. Taken together, proteins from GPCR and PI3K/AKT-mediated growth signaling pathways were more prevalent in E2-stimulated MCF-7 cells, whereas proteins related to apoptosis pathways were more prevalent in E2-stimulated MCF-7:5C cells. The respective connectivity of the pathways is depicted in Figure 4.

Global AIB1 interaction networks

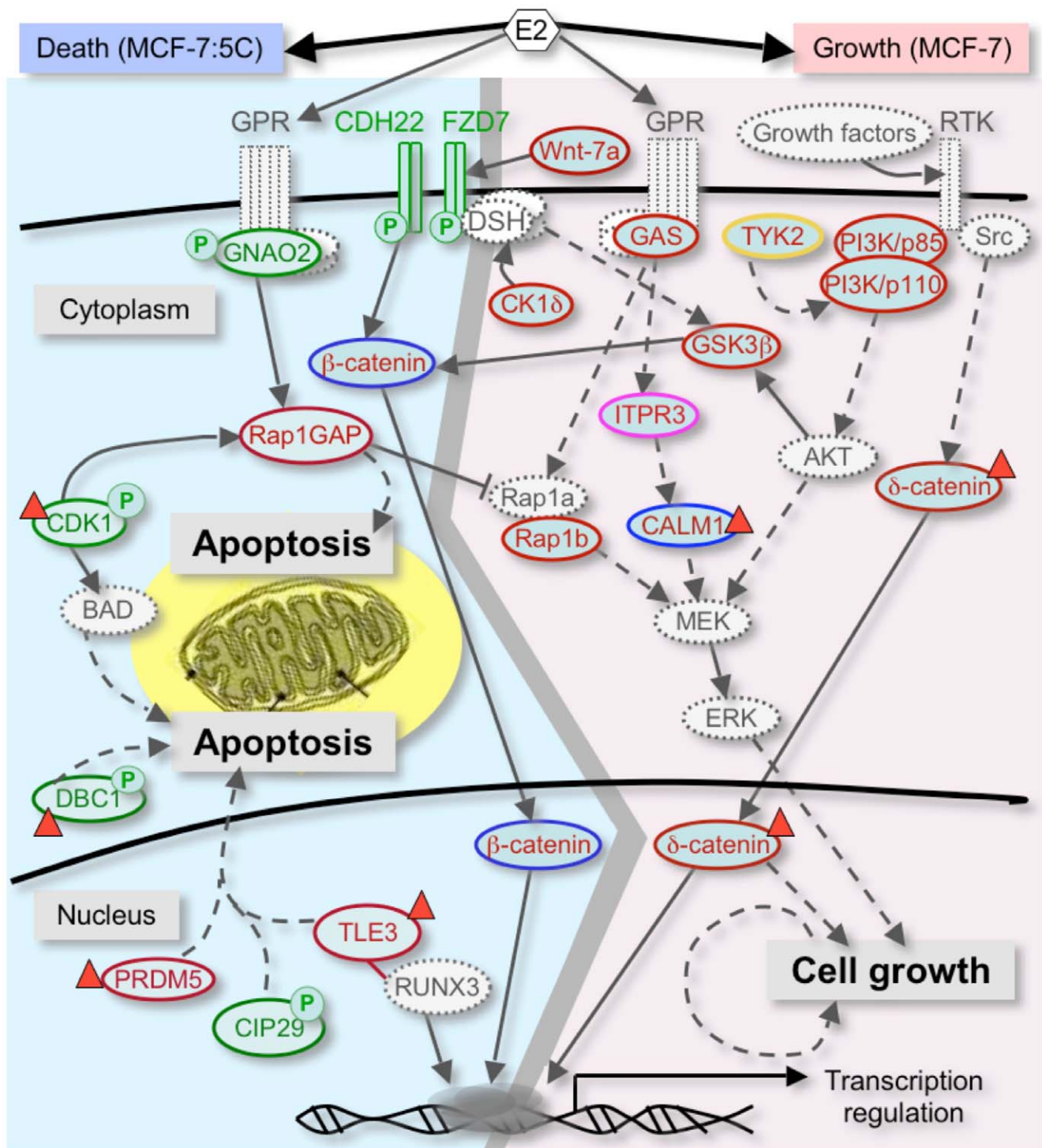
To extract further information from these experimental data, they were linked with an AIB1 interaction network generated from published data [43]. A computational global AIB1 protein interaction network can be constructed from 91 AIB1 interaction partners (first neighbors) based on the literature published since AIB1 was first described in 1997 [37]. These 91 proteins belong to several major functional categories that include transcription, cell communication, developmental processes and cell cycle regulation. The initial network was expanded to secondary interaction neighbors, based on protein-protein interaction data in the public domain. At this level, the network is composed of 1150 proteins, including 21 highly connected nodes that form major hubs (Fig. 5). These hubs include p53, BRCA1, BCL2, ABL1, CDK2, CDK4, EGFR, ER (= ESR1), p38, and MYC (Fig. 5 and S5). Closely related subnetworks of AIB1 (= NCOA3) shown in Figure S5 (*lower panel*), contain four hub proteins: BRCA1, MYC, CDK2 and PSME3. In the present study we identified 26 proteins that are part of the global AIB1 interaction network and function in signal transduction, transcriptional regulation, the cytoskeleton, and the heat shock response.

Eighteen of the proteins experimentally associated with tyrosine-phosphorylated protein complexes are also part of the global AIB1-interaction network. Of these, seven were identified as interacting with AIB1, including CALM1, ACTB, ACTG1, TUBGCP2, MYH9, HSPA1B, and HSPA9. These proteins correspond to interacting hubs, such as CDK4, MYC, PSME3 and CHUK. We conclude that these hubs may participate in the differential cellular responses to E2.

Connection of E2 transcriptome and proteome effects

An interesting question is to what extent the proteomic pathway mapping parallels mRNA expression profiling in MCF-7 and MCF-7:5C cells. Baseline mRNA expression profiles of these cell lines have been posted earlier (GSE10879; ncbi.nlm.nih.gov/). An analysis of mRNA expression regulation after 48 hrs of E2 treatment of the cells was analyzed and published recently [71]. In MCF-7 cells Bcl-2, a major anti-apoptosis gene, was found upregulated by E2 treatment whereas no change of bcl-2 was seen in MCF-7:5C cells. In our analysis Bcl-2 is one of the major hubs in the AIB1 interaction networks (Fig. 5 and S5). On the other hand, the pro-apoptotic Bcl-2 antagonists Bak, Bax and Bim mRNAs were found upregulated 2- to 7-fold after E2 treatment of MCF-7:5C cells whereas no mRNA expression change was seen in the MCF-7 cells. Our analysis shows that upstream regulators of the canonical intrinsic mitochondrial pathway such as RSKs, were identified in the proteomics approach (Fig. 4 and S2).

The most differentially regulated mRNA after E2 treatment was Gadd45beta that was found up-regulated 5-fold in MCF-7:5C cells but down-regulated 5-fold in MCF-7 cells [71]. Gadd45beta was described earlier as a hub of the MAP kinase signaling cascade and



AIB1-IPed: ○ + E2, in given cells ○ + E2, in both cells
○ -/+ E2, in given cells ○ -/+ E2, in both cells
○ ^P ^P pY-IPed + E2, in given cells ▲ CI ≥95% from MS identification
○ Proteins in canonical pathways, but not observed in this study

Figure 4. Pathway overview map of proteins involved in E2-induced cell growth or apoptosis in MCF-7 versus MCF-7:5C breast cancer cells. The thick grey line in the middle provides an arbitrary boundary between the pathways. Anti-AIB1 immunoprecipitated (AIB1-IPed) and anti-pY-immunoprecipitated proteins (pY-IPed) are indicated by red or green circles respectively (keys at the bottom). The blue circled proteins are AIB1-IPed proteins from MCF-7 (CALM1) or MCF-7:5C cells (β -catenin) under both E2-treated and untreated conditions; the purple circled one (ITPR3) is an AIB1-IPed protein from both cells only under E2 treated condition, while the yellow circled one (TYK2) is an AIB1-IPed protein from both cells under both E2 treated and untreated conditions. Proteins circled in grey are from known canonical pathways (e.g. ERK in cell growth or BAD in apoptosis) but not identified here. Solid line arrows indicate direct interactions (e.g. CDK1 phosphorylates Rap1GAP) or translocations (e.g. catenins) of proteins, while dashed arrows indicate indirect actions of proteins (e.g. AKT activate MEK through several steps). Hammer-ended lines indicate inhibitory effects on the target. Detailed pathways are shown in Fig. S1, S2, S3, S4.
doi:10.1371/journal.pone.0020410.g004

connects to relA, the NF κ B p65 subunit (see e.g. Ref. [72]) as well as cell survival in apoptosis resistant cells [73]. We isolated components of GPCR signaling in our proteomics analysis (Fig. 4 and Fig. S1) that can connect to these downstream effectors and can thus serve as trigger mechanisms. Interestingly, GPR30 mRNA was found upregulated in MCF-7:5C cells after estradiol treatment [40] and GPR30 was shown to rapidly transmit non-genomic effects of E2 in breast cancer cells [74]. Overall, the mRNA expression analyses and proteomics data show some interesting convergences especially in apoptotic regulatory pathways which may be functionally relevant as initiators of estradiol-induced apoptosis or cell survival.

Conclusions

The estrogen induced apoptotic response is most strongly associated with early signaling changes in G-protein coupled receptors, PI3 kinase, Wnt and Notch signaling and are integrated here into a global AIB1 signaling network that controls qualitatively distinct responses to estrogen.

Materials and Methods

The overall experimental design

We used combined proteomics and bioinformatics approaches [43] to identify the E2 induced signaling pathways and networks that are associated with AIB1 and/or tyrosine phosphorylated proteins and that differentiate the MCF-7 from MCF-7:5C cells in responses to E2 treatment (Fig. 1A). A single early time point after E2 treatment (2 hrs) was examined to capture signaling events that drive apoptosis or proliferation in these cells. Repeat independent proteomic experiments for each of the 4 experimental conditions and the two different immunoprecipitations were run.

Cell culture

MCF-7 (ATCC) human breast cancer cells and the MCF-7 variant MCF-7:5C [75], which is a clonal variant of MCF-7 derived after longterm estrogen deprivation, were cultured in RPMI-1640 without Phenol Red (Invitrogen) supplemented with 10% FBS, or in RPMI-1640 supplemented with 10% charcoal/

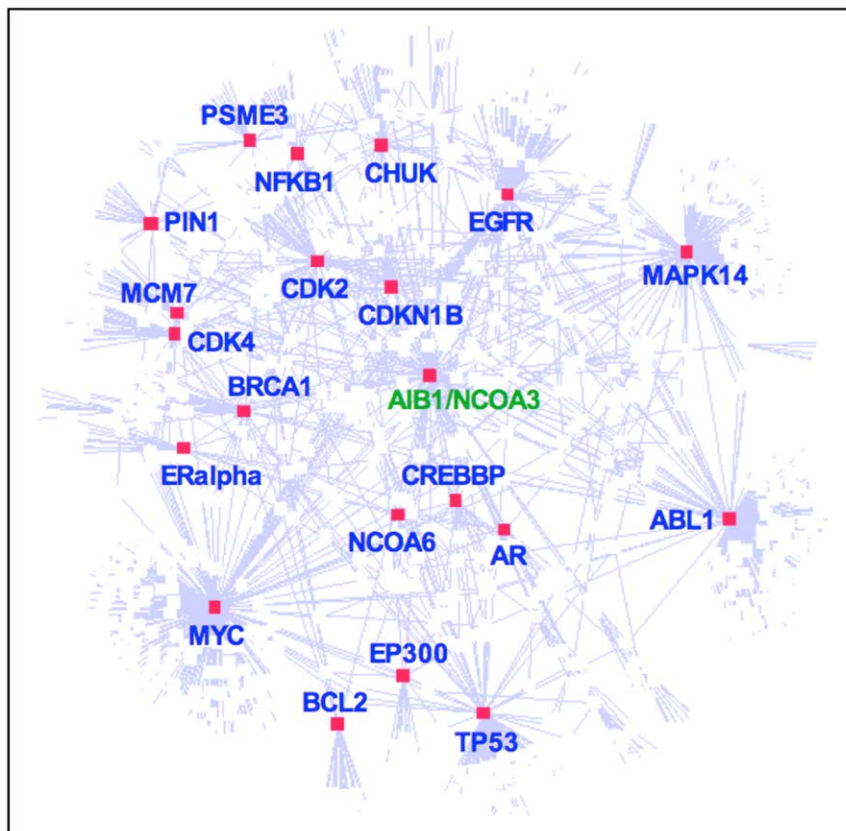


Figure 5. A global AIB1 interaction network showing the major hub proteins. Twenty-one hubs were identified using a cutoff of 20 node degrees. The full names of the respective gene symbols are provided in Table S8. Detailed nodes in the network are shown in Fig. S5.
doi:10.1371/journal.pone.0020410.g005

dextran-stripped FBS (Hyclone) and other supplements, respectively, as described previously [38]. MCF-7 or MCF-7:5C cells deprived of steroid hormones for 2 days were plated at a density of 2,000 and 3,000 cells per well, respectively, in 96-well cell culture plates. One day after plating, cells were treated with E2 (in ethanol) or vehicle (ethanol). To monitor the portion of viable cells after 6 days of growth, the CellTiter-Glo luminescent cell viability assay (Promega) or WST1 colorimetric cell proliferation assay (Roche) were used. Typical readings of baseline growth without E2 were 2.0×10^5 RLU (CellTiter-Glo) or an OD450 of 0.5 (WST1). Data are shown relative to the baseline.

Infection of MCF-7 and MCF-7:5C with lentiviral shRNA expression vectors

Prior to infection, MCF-7 and MCF-7:5C cells were plated at a density of 3×10^5 cells on 10 cm tissue culture dishes. 24 hrs later, cells were infected with lentiviral particles expressing control or AIB1-targeting shRNAs (in pLKO.1). The AIB1(1) shRNA was derived from an siRNA for AIB1 previously described [25], and the AIB1(2) shRNA was from Sigma (TRCN0000019703). The control shRNA used in the experiments is a scrambled sequence described previously [76]. Briefly, 1 ml of lentivirus-containing supernatant was added to 9 ml of growth medium and 8 ng/ml polybrene, and then added to cells for 24 hrs. Medium containing lentivirus was then replaced with growth medium without lentivirus. After two days, cells were treated for 48 hours with 5 μ g/ml puromycin for the selection of lentiviral shRNA expression.

Western blot analysis, immunoprecipitation and protein isolation

Western blot analyses were done as previously described [25], using a monoclonal antibody for AIB1 (SRC3; clone 5E11, Cell Signaling). For the mass spectrometry analysis, protein lysates from cells treated for 2 hours with E2 or vehicle were subjected to immunoprecipitation using gamma-bind G-Sepharose beads and an anti-AIB1 monoclonal antibody (BD Biosciences) as described [77] or an anti-phosphotyrosine monoclonal antibody (4G-10, Millipore). The amount of protein input for immunoprecipitations ranged between 7 mg and 14 mg for each of the experimental conditions with bovine serum albumin used as the standard. It is noteworthy that over a 24 hour period of E2 treatment of cells the AIB1 protein expression levels varied less than 2-fold as illustrated in Figure S6. The immunoprecipitated proteins were separated by denaturing SDS-PAGE on 4–12% Nu-PAGE gels (Invitrogen). After electrophoresis, gels were stained with Coomassie blue overnight and washed with ddH₂O overnight to remove background staining. Stained gels were imaged using a color scanner and visible bands were cut from the gels. The corresponding segments of lanes from the different treatments were also cut for analyses and served as controls. Figure S7 shows a representative set of stained gels with an overlay of the grid of segments harvested for the mass spectrometry analyses.

Mass spectrometry analysis

SDS-PAGE gel slices were subjected to tryptic digest and followed by MS and MS/MS on an ABI MALDI-TOF-TOF. Proteins in the MS or MS/MS analysis were identified based on searches of the Swiss-Prot database using the search engine Mascot 2.0. The Swiss-Prot database searched was based on its 9/24/2007 release (287,050 sequences). The database search parameters used were: 1) enzyme specificity considered, trypsin; 2) number of missed cleavages permitted, 1; 3) fixed modifica-

tion(s), carbamidomethyl (C); 4) variable modification(s), oxidation (M); 5) mass tolerance for precursor ions, 75ppm; and 6) mass tolerance for fragment ions, 0.3 Da. Trypsin autolysis peaks were excluded from the peak list. GPS Explorer (Version 3.0) with default parameter setting was used to generate the peak list from raw data which were submitted to database searches using Mascot. The confidence interval (CI) for the peptide identification was calculated by GPS Explorer. A CI of $\geq 95\%$ (or expectation value ≤ 0.05) was used as a cut off for the high CI proteins.

Bioinformatics Analysis

Protein data filtering. Proteins identified from mass spectrometry were subjected to extensive bioinformatics analysis, including protein data filtering, functional profiling and pathway mapping as described previously [78]. Protein identities from different experimental groups were assigned levels of identification confidence based on statistical processing by GPS Explorer™ of the MASCOT search results. It is commonly known that false negative identification is generated because low-scored proteins may result from factors such as database size, protein abundance and the type of mass spectrometry instrumentation. Therefore, in addition to analyzing the proteomic data based on the prioritized list of proteins with high Confidence Interval (CI; Tables S1, S2), we also used a global approach for pathway mapping on proteins identified at all confidence levels. We provide the identity, CI and spectra of those proteins as well as the reference to the respective pathway figures in Table S3.

We used the following criteria to filter the protein lists. (i) Proteins with MS confidence interval (CI) values smaller than 95% were removed to reduce false-positive results; (ii) Proteins described to be non-specific interactors e.g. HSPA5 and Desmoplakin [79] were removed; (iii) High abundant, non-specific proteins e.g. keratins were removed; (iv) Proteins migrating at an apparent mass in the SDS-PAGE that was different from the calculated mass or the experimentally described mass or the predicted mass were removed. A representative set of Coomassie stained gels after immunoprecipitations is shown in Fig. S7 to illustrate this latter consideration.

Protein annotation, profiling and pathway analysis. The iProXpress bioinformatics system (<http://pir.georgetown.edu/iproxpress>) was used for protein annotation, function and pathway profiling of the proteomics data. The experimental group(s) in which the proteins were identified was annotated for all proteins and integrated into the iProXpress system for direct functional comparison between selected groups, such as cell types, E2 treatment, and experimental repeats. The procedure of using iProXpress system has been described recently [43,78]. The data sets are accessible at <http://pir.georgetown.edu/iproxpress/coe2/>. Pathway mapping and network visualization are assisted with Ingenuity Pathways Analysis (IPA) (www.ingenuity.com) and GeneGO MetaCore (www.GeneGO.com) software tools.

Data mining for known AIB1 interactors. The global AIB1 interaction network refers to a network of genes or proteins that directly or indirectly interact or are functionally associated with AIB1 regardless of cell/tissue types or species in which the interaction occurs. The network is computationally generated based on two sources of data, i.e. the published literature (PubMed) and protein-protein interactions (PPI) available from public databases. A list of AIB1 synonyms included as query terms “AIB1 OR AIB-1 OR NCOA3 OR NCOA-3 OR SRC3 OR SRC-3 OR TRAM1 OR ACTR OR pCIP” to search PubMed and retrieved a total of about 650 papers related to AIB1. Of these papers about 250 papers that contain AIB1 interaction or functional association information were curated, and a total of

91 AIB1 interaction partners were thus obtained. The interaction types in the literature included physical interactions, such as “binding”, “complex”, “interact”, “phosphorylation”, etc., and functional associations, such as “activation”, “correlated expression”, “lead to degradation”, “modulate”, “promoter binding”, “suppression”, etc. These interacting proteins/genes reported for human as well as other species from mouse to *Xenopus*, were mapped to corresponding human orthologs based on UniProtKB database.

The protein/protein interaction (PPI) data annotated in bioinformatics databases were obtained from IntAct database [80], which contains high throughput PPI data from Y2H and IP in addition to literature data. The AIB1 interaction network was constructed based on the binary interactions of the curated 91 AIB1-interacting proteins and those from the PPI database. The network was clustered and filtered, and major hubs were selected using a cutoff of a node degree of 20. Cytoscape open source software was used to display the network for visual examinations.

Supporting Information

Figure S1 Proteins identified in GPCR signaling pathways. Canonical cell growth pathways initiated by GPCR signaling are depicted based on the MetaCore pathway tool of GeneGO. The AIB1- and pY-IPed proteins identified from the study were mapped to the pathway using MetaCore, which were manually re-annotated in the red-lined white boxes with black arrows pointing to the specific protein depictions. The corresponding experimental conditions under which the proteins were identified are indicated at the bottom. Proteins were AIB1-IPed under conditions indicated as A–D, or pY-IPed indicated by “p”. (TIF)

Figure S2 Proteins identified in apoptosis pathways. The canonical intrinsic mitochondrial apoptosis pathway is depicted based the MetaCore pathway tool of GeneGO. Similar to Fig. S3, the anti-AIB1- and pY-IPed proteins identified from the study were mapped to the pathway and were manually re-annotated with red-lined white boxes with the specific protein identified here. (TIF)

Figure S3 Proteins identified in the PI3K/AKT pathway. The canonical PI3K/AKT pathway is depicted based on the Ingenuity pathway tool. AIB1-IPed proteins that were mapped to the canonical pathway are shown as orange-colored shapes in four panels, each representing the same PI3K/AKT pathway with different mapped proteins that were identified from untreated MCF-7 (A) or MCF-7:5C (B) and E2-treated MCF-7 (C) or MCF-7:5C (D) cells. Some proteins in the pathway were manually re-annotated with green-colored box to indicate the specific protein forms identified in this study that correspond to the protein classes represented in the canonical pathway, e.g. JAK refers to the non-receptor type tyrosine kinases, such as TYK2 here. (TIF)

Figure S4 Proteins identified in the Wnt/ β -catenin pathway. The canonical Wnt/ β -catenin pathway is depicted based on the Ingenuity pathway tool. AIB1-IPed proteins that can be mapped to the canonical pathway are shown as orange-colored shapes in four panels, each representing the same Wnt/ β -catenin pathway with different mapped proteins that were identified from untreated MCF-7 (A) or MCF-7:5C (B) and E2-treated MCF-7 (C) or MCF-7:5C (D) cells. Some proteins in the pathway were manually re-annotated with green-colored box to indicate the specific protein forms identified in the experiment that correspond to the classes represented in the canonical pathway, e.g. Wnt refers

to class of Wnt ligands, such as Wnt-4 and Wnt-7a. Some proteins manually labeled with a “P” in red indicate that they were identified as pY-IPed.

(TIF)

Figure S5 AIB1 interaction network. A global AIB1 interaction network (upper) and the selected sub-networks (lower) are shown. The overall topology of the network is displayed with Spring-embedded layout using Cytoscape network visualization software before network clustering (image can be zoomed in to view individual node). Proteins that are identified with high confidence in this study are colored as green (AIB1-IPed), yellow (pY-IPed) or dark brown (both AIB1- and pY-IPed) nodes. Hub proteins that are subsequently clustered with AIB1 in several subnetworks are indicated with arrows (upper). Individual nodes in AIB1-clustered subnetworks are shown in the lower panel, with major functional categories labeled for the hub proteins. (TIF)

Figure S6 Western blot analysis for AIB1. Cells treated with E2 for different times were harvested and Western blot analysis for AIB1 was performed as described in Materials and Methods.

(TIF)

Figure S7 Coomassie stained protein gels after anti-AIB1 or -pY immunoprecipitation (IP). MCF-7 and MCF-7:5C cells were treated or not with E2 for 2 hours, and proteins were extracted for IP. The immunoprecipitated proteins were separated by 4–12% Nu-PAGE, stained, washed with ddH₂O and imaged using a color scanner. The images were magnified and analyzed visually on a screen. After identification, bands were cut from the gels and great care was taken to isolate the same segment of all lanes from the different treatments for a parallel MS analysis. Representative stained gels with the segments to be cut for analysis are indicated. Slices numbered 1–10 or 1–13 were cut from the gels for each segment that showed at least one distinctly regulated protein. Molecular masses of marker proteins are indicated (10–250 kDa). (TIF)

Figure S8 Western blot analysis confirms that FAK1 and TLE3 are immunoprecipitated from E2 treated MCF7:5C cells. MCF-7:5C cells were treated or not with E2 for 2 hours, and proteins were extracted for IP/Western analysis A) Tyrosine-phosphorylated endogenous proteins were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (4G-10, Millipore) and the immunoprecipitate was resolved on SDS-PAGE followed by Western analysis. The input is 5% of the amount of total cell lysates for IP. FAK1 was detected on the blot with an anti-FAK1 antibody (A-17, Santa Cruz). B) AIB1 interacting proteins were immunoprecipitated using an anti-AIB1 monoclonal antibody (BD Biosciences). The input is 5% of the amount of total cell lysates for IP. TLE3 was detected on the blot with a TLE3 antibody (Abcam). (TIF)

Table S1 AIB1-interacting proteins with a CI value of $\geq 95\%$. AIB1-interacting proteins (n = 58) isolated from MCF-7 and MCF-7:5C cells identified by MALDI-MS/MS with a CI value of $\geq 95\%$ are listed and assigned with functional categories. The number of peptides identified and % coverage are in Table S4. Various experimental groups in which AIB1-interacting proteins were identified, are shown in the right side columns (with vertical column names), and the number of total proteins in each group is given in parenthesis. Proteins are arranged by their functional categories (see Fig. 3) and the number of proteins in each experimental group of a given category is also indicated in

the same row of the category. The column furthest to the right shows AIB1-interacting proteins in this study that are also identified as part of the AIB1 protein interaction (int.) network. “X” indicates the presence of a given protein in a given experimental group or in the AIB1 interaction network. Asterisks by the protein accession indicate AIB1-interacting proteins that are also identified in pY complexes (see Table S2). (DOC)

Table S2 Phosphotyrosine complexed proteins with a CI value of $\geq 95\%$. Proteins pulled down with anti-pY in MCF-7 and MCF-7:5C cells identified from MALDI-MS/MS with a CI value of $\geq 95\%$ are listed and assigned with functional categories. The number of peptides identified and % coverage are in Table S5. Various experimental groups in which tyrosine-phosphorylated proteins are identified are shown in the right columns (with vertical column names), and the number of proteins in each group is given in parenthesis. Proteins are arranged by their functional categories (see Fig. 3) and the number of proteins in each experimental group of a given category is also indicated in the same row of the category. “X” indicates the presence of a given protein in a given experimental group or in the AIB1 interaction network. Asterisks by the protein accession indicate IP-pY complexes that are also identified as AIB1-interacting (see Table S1). (DOC)

Table S3 Pathway mapping of proteins identified with a CI < 95%. Proteins are listed alphabetically based on the “gene name” column for anti-AIB1 or anti-pY immunoprecipitated proteins. In the “Experiment” column A to D indicate: A, MCF-7 cells, no E2; B, MCF-7:5C cells, no E2; C, MCF-7 cells, +E2; and D, MCF-7:5C cells, +E2. The “Spec” column references the corresponding mass spectrum for single peptide MS/MS identification in the section “Single peptide spectral data” appended at the end of this table. The “Figures” column indicates in which figure(s) the proteins are depicted, except for a few only discussed in the main text (*text*). In the spectral data section, the underlined C and M in “peptide sequences” column represent fixed (carbamidomethyl) and variable (oxidation) modifications, respectively. *MALDI-TOF-MS generates peptides containing only one charge, and the precursor m/z is thus equal to the precursor mass. NA, not available. (DOC)

Table S4 AIB1-complexed proteins identified by MALDI-TOF-TOF. Proteins were identified based on single MS (MS) or tandem MS (MS/MS) using the search engine Mascot 2.0 from the Swiss-Prot database. Note that the same proteins could be identified under different experimental (“Exp.”) conditions: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. For proteins identified from single peptide MS/MS, spectral data (Spec.) are referenced using the labels (A1–A30) to correspond to those shown in Table S6. All spectra for single peptides shown here are manually inspected, including the one that shows CI of 93% but with good ion fragments. *The % coverage for single peptide MS/MS was only stated if the respective peptide covered $\geq 1\%$ of the protein. The spectra and sequences are in Table S6. (DOC)

References

1. Lippman ME, Bolan G (1975) Oestrogen-responsive human breast cancer in long term tissue culture. *Nature* 256: 592–593.
2. EBC/TCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *The Lancet* 365: 1687–1717.

Table S5 pY-complexed proteins identified by MALDI-TOF-TOF. Proteins were identified based on single MS (MS) or tandem MS (MS/MS) using the search engine Mascot 2.0 from the Swiss-Prot database. Note that the same proteins could be identified under different experimental (“Exp.”) conditions: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. Proteins that were identified more than once from experimental repeats under the same conditions are labeled with * in the “Exp” column. For proteins identified from single peptide MS/MS, spectral data (Spec.) are referenced using the labels (Y1–Y38) to correspond to those shown in Table S7. All spectra for single peptides shown here were manually inspected, including those that show $90\% \leq CI \leq 95\%$ but with good ion fragments. *The % coverage for single peptide MS/MS was only stated if the respective peptide covered $\geq 1\%$ of the protein. The spectra and sequences are in Table S7. (DOC)

Table S6 MS/MS spectra for single peptide identified AIB1-complexed proteins. The “No.” column labels the spectra sequentially as referenced in Table S4. The “Exp.” column indicates the experimental conditions under which the respective protein was identified: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. The underlined C and M in peptide sequences represent fixed (carbamidomethyl) and variable (oxidation) modifications, respectively. *MALDI-TOF-MS generates peptides containing only one charge and the precursor m/z (not shown) is thus equal to the precursor mass. (DOC)

Table S7 MS/MS spectra for single peptide identified pY-complexed proteins. The “No.” column labels the spectra sequentially as referenced in Table S5. The “Exp.” column indicates the experimental conditions under which the protein was identified: A, MCF-7 cells, no E2; B, MCF-7:5C cell, no E2; C, MCF-7 cell, +E2; D, MCF-7:5C cell, +E2. The underlined C and M in peptide sequences represent fixed (carbamidomethyl) and variable (oxidation) modifications, respectively. *MALDI-TOF-MS generates peptides containing only one charge and the precursor m/z (not shown) is thus equal to the precursor mass. (DOC)

Table S8 List of acronyms used. (DOC)

Acknowledgments

We thank Helen Kim and Drs. Annabell Oh, Tyler Lahusen (Georgetown U), and Joan Lewis-Wambi (FCCC) for experimental assistance and Dr. Michael Johnson (Georgetown U) for advice.

Author Contributions

Conceived and designed the experiments: Z-ZH VCJ ATR AW. Performed the experiments: BLK EAA LZ JVL. Analyzed the data: Z-ZH BLK DSR HH CW ATR AW. Wrote the paper: Z-ZH BLK ATR AW.

5. Brauch H, Jordan VC (2009) Targeting of tamoxifen to enhance antitumour action for the treatment and prevention of breast cancer: the 'personalised' approach? *Eur J Cancer* 45: 2274–2283.
6. Fichtner I, Becker M, Zeisig R, Sommer A (2004) In vivo models for endocrine-dependent breast carcinomas: special considerations of clinical relevance. *Eur J Cancer* 40: 845–851.
7. Ariazi EA, Lewis-Wambi JS, Gill SD, Pyle JR, Ariazi JL, et al. (2006) Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant. *The Journal of Steroid Biochemistry and Molecular Biology* 102: 128–138.
8. Lønning PE, Taylor PD, Anker G, Iddon J, Wie L, et al. (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 67: 111–116.
9. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, et al. (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 302: 774–780.
10. Dodds EC, Goldberg L, Lawson W, Robinson R (1938) Estrogenic activity of certain synthetic compounds. *Nature* 141: 247–248.
11. Haddow A, Watkinson J, Paterson E (1944) Influence of synthetic oestrogens upon advanced malignant disease. *BMJ* 2: 393–398.
12. Carter AC, Sedransk N, Kelley RM, Ansfield FJ, Ravdin RG, et al. (1977) Diethylstilbestrol: recommended dosages for different categories of breast cancer patients. Report of the Cooperative Breast Cancer Group. *JAMA* 237: 2079–2078.
13. Jordan VC, Lewis JS, Osipo C, Cheng D (2005) The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. *Breast* 14: 624–630.
14. Song RX, Mor G, Nafolin F, McPherson RA, Song J, et al. (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 β -estradiol. *J Natl Cancer Inst* 93: 1714–1723.
15. Jordan VC, Ford LG (2011) Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-Induced Apoptosis. *Cancer prevention research (Philadelphia, Pa.)*.
16. Macedo LF, Sabnis G, Brodie A (2009) Aromatase inhibitors and breast cancer. *Ann N Y Acad Sci* 1155: 162–173.
17. Musgrove EA, Sutherland RL (2009) Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer* 9: 631–643.
18. Arpino G, Wiechmann L, Osborne C, Schiff R (2008) Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev* 29: 217–233.
19. Lahusen T, Henke R, Kagan B, Wellstein A, Riegel A (2009) The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Research and Treatment* 116: 225–237.
20. Zhao W, Zhang Q, Kang X, Jin S, Lou C (2009) AIB1 is required for the acquisition of epithelial growth factor receptor-mediated tamoxifen resistance in breast cancer cells. *Biochem Biophys Res Commun* 380: 699–704.
21. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, et al. (2003) Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 95: 353–361.
22. Schiff R, Massarweh S, Shou J, Osborne CK (2003) Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 9: 4475–4545.
23. List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, et al. (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. *J Biol Chem* 276: 23763–23768.
24. Font de Mora J, Brown M (2000) AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* 20: 5041–5047.
25. Oh A, List HJ, Reiter R, Mani A, Zhang Y, et al. (2004) The nuclear receptor coactivator AIB1 mediates insulin-like growth factor I-induced phenotypic changes in human breast cancer cells. *Cancer Res* 64: 8299–8308.
26. Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ (2003) Role of the steroid receptor coactivator SRC-3 in cell growth. *Mol Cell Biol* 23: 7742–7755.
27. Lahusen T, Fereshteh M, Oh A, Wellstein A, Riegel AT (2007) Epidermal growth factor receptor tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator, amplified in breast cancer 1. *Cancer Res* 67: 7256–7265.
28. Coste A, Antal M, Chan S, Kastner P, Mark M, et al. (2006) Absence of the steroid receptor coactivator-3 induces B-cell lymphoma. *EMBO J* 25: 2453–2464.
29. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, et al. (2000) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci U S A* 97: 6379–6384.
30. Fereshteh MP, Tilli MT, Kim SE, Xu J, O'Malley BW, et al. (2008) The nuclear receptor coactivator amplified in breast cancer-1 is required for Neu (ErbB2/HER2) activation, signaling, and mammary tumorigenesis in mice. *Cancer Res* 68: 3697–3706.
31. Torres-Arzuayus MI, De Mora JF, Yuan J, Vazquez F, Bronson R, et al. (2004) High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* 6: 263–274.
32. Torres-Arzuayus MI, Yuan J, DellaGatta JL, Lane H, Kung AL, et al. (2006) Targeting the AIB1 oncogene through mammalian target of rapamycin inhibition in the mammary gland. *Cancer Res* 66: 11381–11388.
33. Tilli MT, Reiter R, Oh AS, Henke RT, McDonnell K, et al. (2005) Overexpression of an N-terminally truncated isoform of the nuclear receptor coactivator amplified in breast cancer 1 leads to altered proliferation of mammary epithelial cells in transgenic mice. *Mol Endocrinol* 19: 644–656.
34. Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT (2009) The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Res Treat* 116: 225–237.
35. Xu J, Wu R-C, O'Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. *Nat Rev Cancer* 9: 615–630.
36. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, et al. (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res* 66: 1630–1639.
37. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, et al. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 277: 965–968.
38. Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94: 131–141.
39. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, et al. (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97: 1746–1759.
40. Jordan VC, Lewis-Wambi J, Kim H, Cunliffe H, Ariazi E, et al. (2007) Exploiting the apoptotic actions of estrogen to reverse antihormonal drug resistance in estrogen receptor positive breast cancer patients. *Breast* 16 Suppl 2: S105–113.
41. Bell AW, Deutsch EW, Au CE, Kearney RE, Beavis R, et al. (2009) A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat Methods* 6: 423–430.
42. Aebersold R (2009) A stress test for mass spectrometry-based proteomics. *Nat Methods* 6: 411–412.
43. Hu Z-Z, Huang H, Wu CH, Jung M, Dritschilo A, et al. (2011) Omics-based molecular target and biomarker identification. *Methods Mol Biol* 719: 547–571.
44. Louet JF, Coste A, Amazit L, Tannour-Louet M, Wu RC, et al. (2006) Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. *Proc Natl Acad Sci U S A* 103: 17868–17873.
45. Coste A, Louet JF, Lagouge M, Lerin C, Antal MC, et al. (2008) The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1 α . *Proc Natl Acad Sci U S A* 105: 17187–17192.
46. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, et al. (2000) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci USA* 97: 6379–6384.
47. Long W, Yi P, Amazit L, Lamarca HL, Ashcroft F, et al. (2010) SRC-3 Δ 4 Mediates the Interaction of EGFR with FAK to Promote Cell Migration. *Molecular Cell* 37: 321–332.
48. Deng Q, Huang S (2004) PRDM5 is silenced in human cancers and has growth suppressive activities. *Oncogene* 23: 4903–4910.
49. Brinkmeier ML, Potok MA, Cha KB, Gridley T, Stifani S, et al. (2003) TCF and Groucho-related genes influence pituitary growth and development. *Mol Endocrinol* 17: 2152–2161.
50. Nagahama Y, Ishimaru M, Osaki M, Inoue T, Maeda A, et al. (2008) Apoptotic pathway induced by transduction of RUNX3 in the human gastric carcinoma cell line MKN-1. *Cancer Sci* 99: 23–30.
51. Tong DD, Jiang Y, Li M, Kong D, Meng XN, et al. (2009) RUNX3 inhibits cell proliferation and induces apoptosis by TGF- β -dependent and -independent mechanisms in human colon carcinoma cells. *Pathobiology* 76: 163–169.
52. van Aghoven T, Sieuwerts AM, Meijer-van Gelder ME, Look MP, Smid M, et al. (2009) Relevance of breast cancer antiestrogen resistance genes in human breast cancer progression and tamoxifen resistance. *J Clin Oncol* 27: 542–549.
53. Ahn J, Byeon JJ, Byeon CH, Gronenborn AM (2009) Insight into the structural basis of pro- and antiapoptotic p53 modulation by ASPP proteins. *J Biol Chem* 284: 13812–13822.
54. Liu ZJ, Cai Y, Hou L, Gao X, Xin HM, et al. (2008) Effect of RNA interference of iASPP on the apoptosis in MCF-7 breast cancer cells. *Cancer Invest* 26: 878–882.
55. Sullivan A, Lu X (2007) ASPP: a new family of oncogenes and tumour suppressor genes. *Br J Cancer* 96: 196–200.
56. Zhao Y, Goto K, Saitoh M, Yanase T, Nomura M, et al. (2002) Activation function-1 domain of androgen receptor contributes to the interaction between subnuclear splicing factor compartment and nuclear receptor compartment. Identification of the p102 U5 small nuclear ribonucleoprotein particle-binding protein as a coactivator for the receptor. *J Biol Chem* 277: 30031–30039.
57. Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, et al. (2010) Global characterization of transcriptional impact of the SRC-3 coregulator. *Mol Endocrinol* 24: 859–872.
58. Jordan JD, Carey KD, Stork PJ, Iyengar R (1999) Modulation of rap activity by direct interaction of Galphao with Rap1 GTPase-activating protein. *J Biol Chem* 274: 21507–21510.

59. Zhang J, Liu J, Yu C, Lin A (2005) BAD Ser128 is not phosphorylated by c-Jun NH2-terminal kinase for promoting apoptosis. *Cancer Res* 65: 8372–8378.
60. Klumpp S, Krieglstein J (2002) Serine/threonine protein phosphatases in apoptosis. *Curr Opin Pharmacol* 2: 458–462.
61. Fernando RI, Wimalasena J (2004) Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. *Mol Biol Cell* 15: 3266–3284.
62. Roux PP, Richards SA, Blenis J (2003) Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol Cell Biol* 23: 4796–4804.
63. Bundy DL, McKeithan TW (1997) Diverse effects of BCL3 phosphorylation on its modulation of NF-kappaB p52 homodimer binding to DNA. *J Biol Chem* 272: 33132–33139.
64. Mathas S, Johrens K, Joos S, Lietz A, Hummel F, et al. (2005) Elevated NF-kappaB p50 complex formation and Bcl-3 expression in classical Hodgkin, anaplastic large-cell, and other peripheral T-cell lymphomas. *Blood* 106: 4287–4293.
65. Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, et al. (2009) The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. *Proc Natl Acad Sci USA* 106: 15732–15737.
66. Zimmer A, Reynolds K (1994) Gene targeting constructs: effects of vector topology on co-expression efficiency of positive and negative selectable marker genes. *Biochem Biophys Res Commun* 201: 943–949.
67. Hoffman BG, Zavaglia B, Beach M, Helgason CD (2008) Expression of Groucho/TLE proteins during pancreas development. *BMC Dev Biol* 8: 81.
68. Liu Y, Dehni G, Purcell KJ, Sokolow J, Carcangiu ML, et al. (1996) Epithelial expression and chromosomal location of human TLE genes: implications for notch signaling and neoplasia. *Genomics* 31: 58–64.
69. Cuevas IC, Slocum AL, Jun P, Costello JF, Bollen AW, et al. (2005) Meningioma transcript profiles reveal deregulated Notch signaling pathway. *Cancer Res* 65: 5070–5075.
70. Hiraie H, Wada-Hiraie O, Nakagawa S, Koyama S, Miyamoto Y, et al. (2010) Identification of DBC1 as a transcriptional repressor for BRCA1. *Br J Cancer* 102: 1061–1067.
71. Lewis-Wambi JS, Jordan VC (2009) Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? *Breast Cancer Res* 11: 206.
72. Papa S, Zazzeroni F, Bubici C, Jayawardena S, Alvarez K, et al. (2004) Gadd45 β mediates the NF- κ B suppression of JNK signalling by targeting MKK7/JNKK2. *Nat Cell Biol* 6: 146–153.
73. Engelmann A, Speidel D, Bornkamm GW, Deppert W, Stocking C (2008) Gadd45 beta is a pro-survival factor associated with stress-resistant tumors. *Oncogene* 27: 1429–1438.
74. Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, et al. (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* 70: 1184–1194.
75. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 90: 77–86.
76. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098–1101.
77. Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, et al. (2008) Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. *Mol Cell Biol* 28: 6580–6593.
78. Hu Z-Z, Huang H, Cheema A, Jung M, Dritschilo A, et al. (2008) Integrated Bioinformatics for Radiation-Induced Pathway Analysis from Proteomics and Microarray Data. *Journal of proteomics & bioinformatics* 1: 47–60.
79. Han SJ, Jung SY, Malovannaya A, Kim T, Lanz RB, et al. (2006) A scoring system for the follow up study of nuclear receptor coactivator complexes. *Nucl Recept Signal* 4: e014.
80. Kerrien S, Alam-Faruque Y, Aranda B, Bancarz I, Bridge A, et al. (2007) IntAct—open source resource for molecular interaction data. *Nucleic Acids Res* 35: D561–565.

The Selective Estrogen Receptor Modulator Bazedoxifene Inhibits Hormone-Independent Breast Cancer Cell Growth and Down-Regulates Estrogen Receptor α and Cyclin D1^[S]

Joan S. Lewis-Wambi, Helen Kim, Ramona Curpan, Ronald Grigg, Mohammed A. Sarker, and V. Craig Jordan

Women's Cancer Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania (J.S.L.W.); Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC (V.C.J., H.K.); Institute of Chemistry, Romanian Academy, Timisoara, Romania (R.C.); and School of Chemistry, University of Leeds, Leeds, United Kingdom (R.G., M.A.S.)

Received March 14, 2011; accepted July 7, 2011

ABSTRACT

Bazedoxifene (BZA) is a third-generation selective estrogen receptor modulator (SERM) that has been approved for the prevention and treatment of postmenopausal osteoporosis. It has antitumor activity; however, its mechanism of action remains unclear. In the present study, we characterized the effects of BZA and several other SERMs on the proliferation of hormone-dependent MCF-7 and T47D breast cancer cells and hormone-independent MCF-7:5C and MCF-7:2A cells and examined its mechanism of action in these cells. We found that all of the SERMs inhibited the growth of MCF-7, T47D, and MCF-7:2A cells; however, only BZA and fulvestrant (FUL) inhibited the growth of hormone-independent MCF-7:5C cells. Cell cycle analysis revealed that BZA and FUL induced G₁ blockade in MCF-7:5C cells; however, BZA down-regulated cyclin D1, which was constitutively overexpressed in these cells, whereas

FUL suppressed cyclin A. Further analysis revealed that small interfering RNA knockdown of cyclin D1 reduced the basal growth of MCF-7:5C cells, and it blocked the ability of BZA to induce G₁ arrest in these cells. BZA also down-regulated estrogen receptor- α (ER α) protein by increasing its degradation and suppressing cyclin D1 promoter activity in MCF-7:5C cells. Finally, molecular modeling studies demonstrated that BZA bound to ER α in an orientation similar to raloxifene; however, a number of residues adopted different conformations in the induced-fit docking poses compared with the experimental structure of ER α -raloxifene. Together, these findings indicate that BZA is distinct from other SERMs in its ability to inhibit hormone-independent breast cancer cell growth and to regulate ER α and cyclin D1 expression in resistant cells.

Introduction

Bazedoxifene acetate (BZA) is a new third-generation selective estrogen receptor modulator (SERM) (Silverman et al., 2008) that is approved in Europe and is under regulatory review in the United States for the prevention and treatment of postmenopausal osteoporosis. In phase III clinical trials (Miller et al., 2008; Archer et al., 2009; Pinkerton et al., 2009)

BZA (20 or 40 mg/daily) has been shown to prevent bone loss and to reduce bone turnover in postmenopausal women at risk for osteoporosis, with a favorable endometrial, ovarian, and breast safety profile. BZA also significantly reduces the risk of new vertebral fractures in postmenopausal women with osteoporosis compared with placebo (Silverman et al., 2008). In addition, recent studies indicate that BZA combined with conjugated estrogens relieves hot flashes and improves vulvovaginal atrophy and its symptoms (Kagan et al., 2010).

BZA is an indole-based ER ligand with unique structural characteristics with respect to tamoxifen (TAM) and raloxifene (RAL). It was assembled by using RAL as a template and substituting an indole ring for the benzothiophene core (Miller et al., 2001; Komm et al., 2005). BZA binds to both ER α and ER β , with a slightly higher affinity for ER α ; how-

This work was supported by the National Institutes of Health National Cancer Institute [Grants K01-CA12005101A2, P30-CA006927]; the American Cancer Society [Grant IRG-9202714]; the Department of Defense [Grant BC050277]; and the National University Research Council of Romania-CNC-SIS-UEFISCU [Grant PN-II-PCE-ID1268].

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.111.072249.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: BZA, bazedoxifene acetate; ER, estrogen receptor; SERM, selective estrogen receptor modulator; TAM, tamoxifen; RAL, raloxifene; E2, 17 β -estradiol; FUL, fulvestrant; siRNA, small interfering RNA; 4OHT, 4-hydroxytamoxifen; ENDOX, endoxifen; Luc, luciferase; ERE, estrogen response element; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; IFD, Induced Fit Docking; SRC, steroid receptor coactivator; ICI 182,780, fulvestrant; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; PDB, Protein Data Bank.

ever, it is less ER α -selective than RAL, with an affinity for ER α that is approximately 10-fold lower than 17 β -estradiol (E2) (Miller et al., 2001). ER α is a well studied member of the steroid/nuclear receptor family of transcription regulators. ER α acts in the nucleus to regulate gene expression by binding to estrogen response elements (EREs) and related DNA sequences and through association with transcription factors bound at SP1 and AP-1 DNA binding sites. In response to high-affinity estrogen binding, ER α dimerizes, binds to ERE DNAs, and undergoes a conformational change in the ligand binding domain that facilitates the recruitment of coactivators. In contrast, antagonist-occupied ER α recruits corepressors. Although previous studies have reported that BZA antagonizes E2-dependent MCF-7 breast cancer cell proliferation *in vitro* (Komm et al., 2005), little is known about the actions of BZA on ER α expression and functionality. In addition, not known is whether BZA has antitumor activity in breast cancer cells that have acquired resistance to endocrine therapies.

We have reported previously the development of two ER α -positive human breast cancer cell lines, MCF-7:5C (Jiang et al., 1992; Lewis et al., 2005a) and MCF-7:2A (Pink et al., 1995; Lewis-Wambi et al., 2008b), that were clonally selected from hormone-dependent MCF-7 breast cancer cells after long-term (>1 year) estrogen deprivation. An interesting phenotype of MCF-7:5C and MCF-7:2A cells is that, unlike MCF-7 cells, which require estrogen to grow and are inhibited by antiestrogens, they do not require estrogen to grow and they undergo apoptosis when exposed to physiological levels of E2 (Lewis et al., 2005a; Jordan, 2008; Lewis-Wambi et al., 2008b). However, the effects of SERMs on MCF-7:5C and MCF-7:2A cells have not been fully examined. In this study, we investigated the effects of BZA, 4-hydroxytamoxifen (4OHT), endoxifen (ENDOX), RAL, and the pure antiestrogen fulvestrant (ICI 182,780) on the growth of MCF-7:5C and MCF-7:2A breast cancer cells and determined the mechanism of action of BZA in these cells. We found that all of the SERMs inhibited E2-stimulated MCF-7 and T47D breast cancer cell growth; however, only BZA and FUL significantly inhibited the hormone-independent growth of MCF-7:5C cells. The inhibitory effect of BZA was associated with cell cycle arrest and cyclin D1 and ER α down-regulation, which was reversed by small interfering RNA (siRNA) knockdown of cyclin D1 and ER α . It is noteworthy that we found that FUL also inhibited MCF-7:5C cell growth; however, this compound partially down-regulated cyclin D1. Together, these data show that BZA is distinct from the other members of the SERM family in its ability to inhibit the growth of breast cancer cells that are resistant to long-term estrogen deprivation.

Materials and Methods

Reagents and Cell Culture. E2, 4OHT (the active metabolite of TAM), and *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG132) were purchased from Sigma-Aldrich (St. Louis, MO). Fulvestrant (ICI 182,780, Faslodex) was a generous gift from Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). ENDOX was a kind gift from Dr. James Ingle of the Mayo Clinic (Rochester, MN). RAL was a generous gift from Lilly Research Laboratories (Indianapolis, IN). BZA was synthesized by authors R.G. and M.A.S. using a protocol described previously (Miller et al., 2001). All of the compounds were dissolved in 100% ethanol except for MG132, which was dissolved in dimethyl sulfoxide. The compounds were added to the medium such that the total solvent concentration was never higher than 0.1%. An

untreated group served as a control. The chemical structures of the compounds used in this study have been cited before (Komm et al., 2005; Jordan, 2007, 2009) and are shown in Supplemental Fig. 1.

MCF-7:WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E2 (Pink and Jordan, 1996), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in estrogenized medium (phenol red RPMI 1640 plus 10% fetal bovine serum), but 3 days before all experiments, they were cultured in steroid-free media as described previously (Pink and Jordan, 1996; Lewis et al., 2005a,b). MCF-7:5C (Jiang et al., 1992; Lewis et al., 2005a,b), and MCF-7:2A cells (Pink and Jordan, 1996; Lewis-Wambi et al., 2008b) were derived from the MCF-7 line by growth in estrogen-free media and two rounds of limiting dilution cloning and were maintained in phenol red-free RPMI 1640 medium containing 10% 3 \times dextran-coated charcoal-treated fetal bovine serum. MC2 cells were derived by stably transfecting ER-negative MDA-MB-231 breast cancer cells with the wild-type ER α (Jiang and Jordan, 1992), and these cells were grown in phenol red-free minimal essential medium supplemented with 5% 3 \times dextran-coated charcoal-treated calf serum, 0.5 mg/ml G-418. All cell culture reagents were from Invitrogen (Carlsbad, CA).

Cell Proliferation Assay. These procedures have been reported previously (Lewis et al., 2005; Lewis-Wambi et al., 2008). In brief, MCF-7 and T47D cells were grown in fully estrogenized medium, whereas MCF-7:5C and MCF-7:2A cells were grown in nonestrogenized media. Cells were seeded in 24-well plates (30,000/well), and after overnight incubation, cells were treated with various concentrations of the tested compounds for 7 days. Media were changed on days 3 and 5, the experiment was ended on day 7, and the DNA content of the cells was determined as described previously (Labarca and Paigen, 1980) using a Fluorescent DNA Quantitation kit (Bio-Rad Laboratories, Hercules, CA). Cell proliferation was also determined by cell counting using a hemocytometer.

Western Blot Analyses. Immunoblotting was performed using 30 μ g of protein per well as described previously (Lewis et al., 2005a). Membranes were probed with primary antibodies against ER α , progesterone receptor, cyclin A, cyclin B1, or cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) with β -actin (AC-15; Sigma-Aldrich) used to standardize loading. The appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize the stained bands with an enhanced chemiluminescence visualization kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Bands were quantitated by densitometry using ImageQuant (GE Healthcare), and densitometric values were corrected for loading control.

Cell Cycle Analyses. MCF-7 and MCF-7:5C cells were treated with E2 or BZA for 24 and 48 h and then fixed using ice-cold 70% ethanol. Cell cycle distribution was determined by propidium iodide staining using a fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) as described previously (Ariazi et al., 2010). Data were analyzed using FlowJo 7.2.5 for Windows (Tree Star, Ashland, OR).

Knockdown of ER α and Cyclin D1 by siRNA. MCF-7:5C cells were seeded at 10⁵ cells/well in a 24-well plate overnight and then transfected with 100 nM nonspecific, ER α , or cyclin D1 siRNA (Dharmacon RNA Technologies, Lafayette, CO) using Lipofectamine 2000 (Invitrogen), as described previously (Lewis et al., 2005a). Transfected cells were either harvested for Western blot analysis or reseeded for cell growth or cell cycle analysis.

Quantitative Real-Time PCR. The detail procedures have been reported previously (Lewis et al., 2005). MCF-7 and MCF-7:5C cells were treated with either E2 (10⁻⁹ M) or BZA (10⁻⁷ M) for 48 h, and total RNA was isolated and then reverse-transcribed to cDNA using the SuperScript II RNase H reverse transcriptase system (Invitrogen). Aliquots of the cDNA were combined with the SYBR green kit (Superarray) and primers and assayed in triplicate by quantitative PCR over 40 cycles using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA) as described previously

(Lewis et al., 2005a). Quantitation was done using the comparative CT method with 18S rRNA as the normalization gene, as described previously (Lewis-Wambi et al., 2008a). PCR primer sequences used were as follow: ER α forward, 5'-GGAGGGCAGGGGTGAA-3'; ER α reverse, 5'-GGCCAG-GCTGTCTTC TTAGA-3'; cyclin D1 forward, 5'-TCCTGTGCTGCGA AGTGGAAC-3'; cyclin D1 reverse, 5'-AAATCGTGC GG GTCATTCG-3'; pS2 forward, 5'-GAGGCCACAGACAGACGTG-3'; and pS2 reverse, 5'-CCCTGCAGAAGTGTCTAAAATTCA-3'.

Transient Transfections and Luciferase Assays. Cells were cultured in estrogen-free RPMI 1640 media for 48 h before transfection. On the day of the experiment, cells were seeded in estrogen-free media at a density of 1.5×10^5 cells per well in 24-well plates. After 24 h, cells were transfected with the firefly luciferase reporter plasmid pERE(5 \times)TA-flLuc (containing five copies of a consensus ERE and a TATA-box driving firefly luciferase) and the pTA-srLuc *Renilla reniformis* luciferase plasmid (containing a TATA-box element driving *R. reniformis* luciferase) (Promega, Madison, WI) using LT1 (Mirus) transfection reagent, according to the manufacturer's protocol. After 24 h, transfection reagents were removed, and fresh media were added. Cells were then treated with ethanol (vehicle), 10^{-9} M E2, 10^{-8} M BZA, or E2 + BZA combined for 24 h. At the indicated time point, cells were washed, lysed, and ERE luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. Samples were then read on a Mithras MB540 luminometer (Berthold Technologies, Oak Ridge, TN).

For the cyclin D1 promoter assay, MCF-7:5C cells were transiently transfected with the full-length cyclin D1 promoter plasmid (–1745CD1-LUC) as described previously (Lewis et al., 2005c,d). The full-length cyclin D1 plasmid (–1745CD1-LUC) (Albanese et al., 1995) was a gift from Dr. Richard Pestell (Thomas Jefferson Kimmel Cancer Center, Philadelphia, PA).

Molecular Modeling. The molecular modeling performed in this study has been described previously (Maximov et al., 2010). In brief, the coordinates for the agonist and antagonist conformations of human ER α ligand binding domain cocrystallized with E2, RAL, and 4OHT were extracted from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (Berman et al., 2000). Entries 1gwr for E2 (Wärnmark et al., 2002), 1err for RAL (Brzozowski et al., 1997), and 3ert for 4OHT (Shiau et al., 1998) were selected for further modeling, and these structures were prepared for docking using the Protein Preparation Workflow (Friesner et al., 2004; Guallar et al., 2004) implemented in Schrödinger suite and accessible from within the Maestro 8.5 program (Schrödinger, Cambridge, MA). To study the molecular basis of interaction of bazedoxifene in the antagonist conformation of ER α , the ligands were docked into the binding site of the receptor cocrystallized with RAL (PDB code 1err). For comparison reasons, RAL was also docked in its native protein structure.

The input geometries of the ligands were generated with CORINA (online demo, http://www.molecular-networks.com/online_demos/corina_demo) and were further prepared for docking using the LigPrep2.2 utility (Friesner et al., 2004; Guallar et al., 2004). The prepared structure of ER α cocrystallized with RAL was used to generate the scoring grid for docking simulations. A grid box of $26 \times 26 \times 26$ Å³ centered on the ligand was created, using the default parameters and without constraints.

Flexible ligand docking simulations were carried out with Glide 5.0 (Friesner et al., 2004; Guallar et al., 2004) using the default settings, and the best 10 poses for each ligand were evaluated using Glide (Schrödinger) in Standard-Precision (GlideSP) and Extra-Precision (GlideXP) mode. The results obtained from the docking runs were compared, and GlideXP docking poses were selected for analysis.

Statistical Analysis. All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean \pm S.D. Statistical significances between vehicle treatment versus drug treatment were determined by one-way anal-

ysis of variance and the Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

BZA Inhibits the Growth of Hormone-Independent MCF-7:5C and MCF-7:2A Breast Cancer Cells. We first compared the growth characteristics of hormone-dependent MCF-7 and T47D breast cancer cells with those of long-term estrogen deprived MCF-7:5C and MCF-7:2A cells in the presence of E2. Cells were grown in estrogen-free media and then treated with 10^{-14} M to 10^{-8} M E2 for 7 days, and cellular DNA was measured as an index of growth. In parallel, cells were also treated with 10^{-9} M E2 for 2 to 12 days and then harvested and counted using a hemocytometer. Figure 1A shows that E2 treatment stimulated the growth of MCF-7 and T47D cells in a concentration-dependent manner with maximum stimulation at 10^{-9} M, whereas in MCF-7:5C and MCF-7:2A cells, E2 treatment had the opposite effect causing either complete growth inhibition in MCF-7:5C cells or partial growth inhibition in MCF-7:2A cells. This finding is consistent with our previous work (Lewis et al., 2005a; Lewis-Wambi et al., 2008b), which showed that physiological concentrations of E2 induced programmed cell death (apoptosis) in MCF-7:5C and MCF-7:2A cells through activation of the mitochondrial death pathway and suppression of glutathione synthesis, respectively. Specifically, we found that E2 induced apoptosis in MCF-7:5C cells by activating proapoptotic proteins Bax, Bak, Bim, and p53 and by suppressing antiapoptotic proteins. E2 also down-regulated survival proteins such as nuclear factor- κ B, phospho-Akt, and Her2/neu, which were overexpressed in MCF-7:5C cells. In contrast, we found that MCF-7:2A cells underwent apoptosis after 10 to 12 days of E2 treatment and that these cells expressed elevated levels of the antioxidant glutathione as a result of overexpression of glutathione synthetase and glutathione peroxidase 2, the two main enzymes involved in glutathione synthesis. By selectively blocking the glutathione pathway in MCF-7:2A cells, we were able to sensitize these cells to E2-induced apoptosis, which was mediated by activation of the c-Jun NH₂-terminal kinase signaling pathway.

Next, we determined the inhibitory effects of BZA and other SERMs (see Supplemental Fig. 1 for chemical structures) on MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. For experiments, MCF-7 and T47D cells were grown in fully estrogenized media, and MCF-7:5C and MCF-7:2A cells were grown in estrogen-free media and then treated with 10^{-12} to 10^{-6} M BZA, RAL, FUL, 4OHT, or ENDOX for 7 days, and cellular DNA was measured as an index of growth. Figure 1B shows that all of the tested SERMs along with the pure antiestrogen FUL inhibited E2-stimulated growth in MCF-7 and T47D cells and hormone-independent growth in MCF-7:2A cells in a concentration-dependent manner; however, in MCF-7:5C cells, only BZA and FUL inhibited the growth of these cells with no effects observed with RAL, 4OHT, or ENDOX. BZA reduced the growth of MCF-7:5C cells in a concentration-dependent manner, causing an 80% reduction at 10^{-8} M, whereas FUL reduced the growth by 55% at a similar concentration.

BZA Down-Regulates ER α Protein in MCF-7:5C and MCF-7:2A Cells. Because BZA dramatically reduced the growth of MCF-7:5C cells, we next determined whether BZA

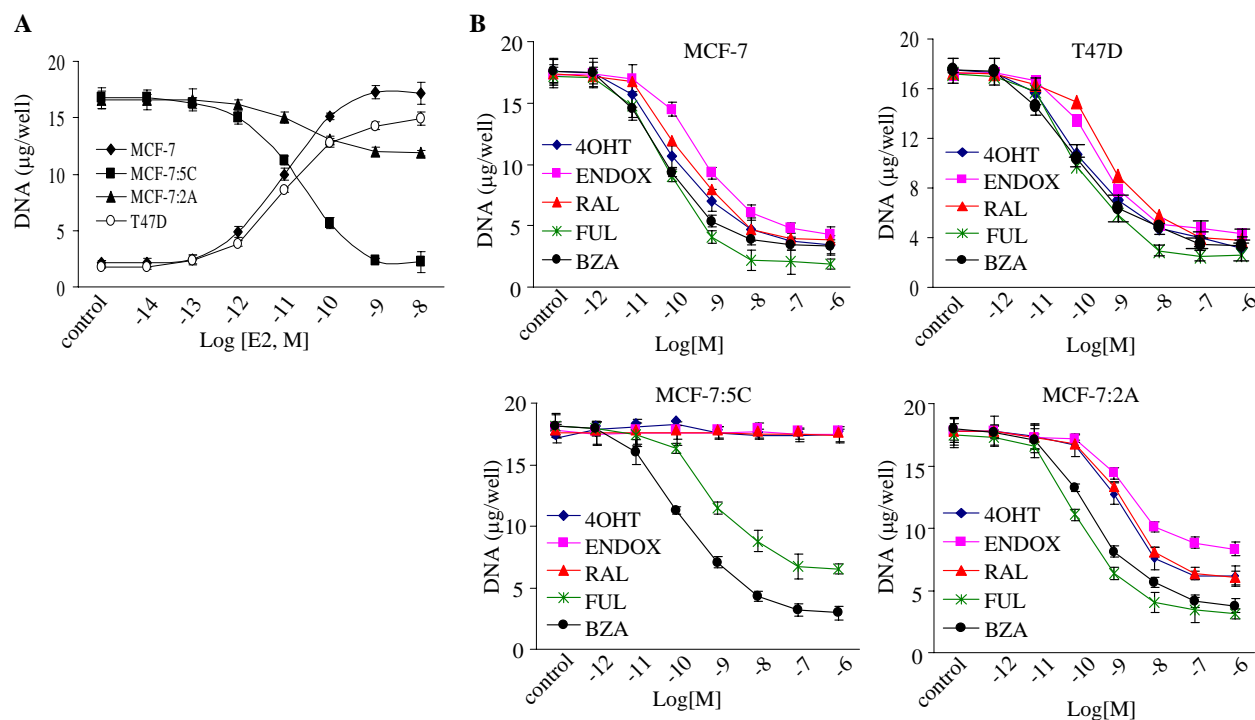


Fig. 1. Effects of E2 and SERMs on the growth of hormone-dependent MCF-7 and T47D cells versus hormone-independent MCF-7:5C and MCF-7:2A cells. A, MCF-7 and T47D cells were grown in phenol red-free RPMI medium supplemented with 10% charcoal-stripped fetal bovine serum for 3 days before the start of the experiment. On the day of the experiment, all cell lines were seeded in phenol red-free RPMI medium supplemented with 10% charcoal-stripped fetal bovine serum at 30,000 per well in 24-well dishes and after 24 h were treated with 10⁻¹⁴ to 10⁻⁸ M E2 for 7 days, with retreatment every other day. At the conclusion of the experiment, cells were harvested, and proliferation was assessed as cellular DNA mass (in micrograms per well) using a DNA quantitation kit. B, the effects of antihormones on the growth of hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. Cells were seeded as described above, except MCF-7 and T47D cells were grown in fully estrogenized media and then treated with 10⁻¹² to 10⁻⁶ M FUL, BZA, RAL, 4OHT, or ENDOX for 7 days with retreatment on alternate days. Proliferation was assessed as cellular DNA mass (in micrograms per well) as described under *Materials and Methods*. Each point represents the mean of three determinations \pm S.E.M.

had actions similar to that of 4OHT or FUL at the level of ER α stability/degradation. We treated MCF-7:5C, MCF-7:2A, MCF-7, and T47D cells with 10⁻⁹ M E2 or 10⁻⁷ M FUL, 4OHT, RAL, or BZA for 24 h and monitored ER α protein level. As shown in Fig. 2A, ER α protein was highly expressed in MCF-7:5C and MCF-7:2A cells compared with MCF-7 and T47D cells and treatment with BZA markedly down-regulated ER α protein in MCF-7:5C and MCF-7:2A cells; however, it did not significantly reduce ER α levels in MCF-7 and T47D cells. The ability of BZA to down-regulate ER α in MCF-7:5C and MCF-7:2A cells was greater than that of RAL and almost comparable with that of the pure antiestrogen FUL, which strongly down-regulated ER α in all of the cell lines. E2 treatment also markedly down-regulated ER α protein in all of the cell lines including MCF-7:5C (Fig. 2A); however, 4OHT stabilized ER α against degradation in MCF-7 and T47D cells, as reported previously (Pink and Jordan, 1996), with marginal stabilization observed in MCF-7:5C and MCF-7:2A cells (Fig. 2A). We also examined the effect of the tamoxifen metabolite ENDOX on ER α expression in the different cell lines and found that endoxifen did not down-regulate ER α in any of the tested cell lines (Supplemental Fig. 2). Our finding differs from that of Wu et al. (2009), who reported that endoxifen degrades ER α in breast cancer cells.

We also performed dose-response studies in MCF-7, MCF-7:5C, and MCF-7:2A cells to determine the optimal concentration at which BZA down-regulated ER α protein. Figure 2B

showed that BZA reduced ER α protein level in MCF-7:5C cells in a concentration-dependent manner with maximum inhibition at 10⁻⁶ M, whereas in MCF-7 and MCF-7:2A cells, BZA only marginally reduced ER α protein in these cells. It is noteworthy that the inhibitory effect of BZA on ER α protein was less pronounced than that observed with E2 or FUL, which almost completely reduced ER α protein level in MCF-7:5C cells. Time course studies revealed that BZA down-regulated ER α protein as early as 2 h after treatment with maximum suppression at 24 h (Fig. 2C, top). BZA also down-regulated ER α mRNA in MCF-7:5C cells to a level similar to that observed with E2 and FUL (Fig. 2C, bottom). To show that the decreased ER α protein by BZA was due to protein degradation, we used MG132 to inhibit the proteasome in MCF-7:5C and MCF-7 cells. We found that inhibition of proteasome activity completely blocked ER α degradation by BZA and E2 with partial reversal with fulvestrant (Fig. 2D). We further determined whether BZA might affect ER α protein expression by inhibiting its synthesis. We treated MCF-7:5C cells with 0.5 to 5 μ M cycloheximide for 4 h to address this question. The impact of cycloheximide on ER α protein expression was much less dramatic than that of BZA (data not shown), which suggest that BZA-induced down-regulation of ER α protein is not likely to involve protein synthesis inhibition. Together, these data show that BZA differs from the other SERMs in its ability to regulate cell growth and ER α protein expression in MCF-7:5C cells.

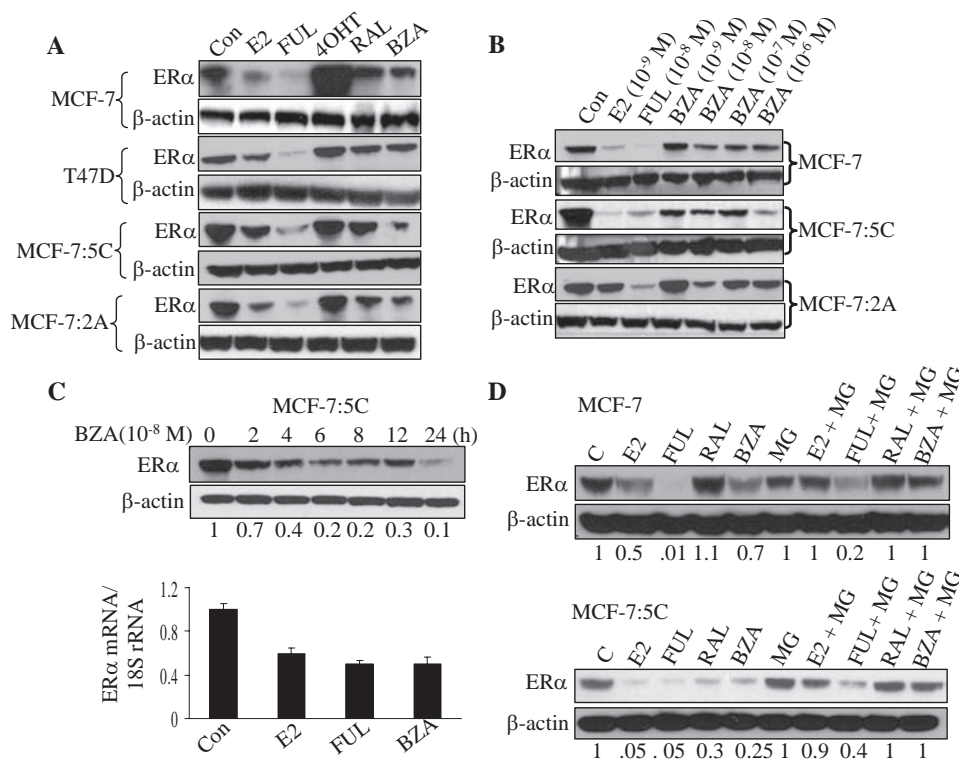


Fig. 2. Effects of SERMs on ER α expression and stability in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. **A**, Western blot analysis of ER α protein levels in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells in response to 24-h treatment with 10^{-9} M E2 or 10^{-7} M FUL, 4OHT, RAL, or BZA. β -actin was used as a loading control. **B**, Western blot analysis of ER α protein levels in MCF-7, MCF-7:5C, and MCF-7:2A cells after treatment with 10^{-9} to 10^{-6} M BZA for 24 h. For comparison, cells were also treated with 10^{-9} M E2 or 10^{-8} M FUL. **C**, Western blot analysis of ER α protein levels in MCF-7:5C cells in response to 10^{-8} M BZA treatment over a 24-h time period. Quantitated protein levels were normalized to β -actin. Densitometric quantitation relative to the control is shown on the bottom of the immunoreactive bands. Also shown is ER α mRNA levels in MCF-7:5C cells after treatment with E2 (10^{-9} M), FUL (10^{-8} M), or BZA (10^{-8} M) for 24 h. The amount of ER α mRNA was determined by real-time RT-PCR and normalized to the internal control 18S rRNA. Each data point represents the average of four biological replicates from three independent experiments. **D**, Western blot analysis of ER α protein levels in MCF-7 and MCF-7:5C cells pretreated with the proteasome inhibitor MG132 (4 μ M) for 4 h and then treated as indicated for 8 h. β -Actin levels are shown as protein loading controls. Each point represents the mean of three determinations \pm S.E.M.

BZA Inhibits ER α Transcriptional Activity in MCF-7:5C Cells. To determine whether BZA blocks ER α function, we next examined the transcriptional activation of an ERE in MCF-7, T47D, MCF-7:5C, and MCF-7:2A cells. Cells were transiently transfected with a 5 \times ERE-luciferase reporter plasmid and treated with 10^{-10} M E2, 10^{-8} M BZA, or E2 + BZA for 24 h. The results of these studies showed that basal ERE activity was elevated 5-fold in MCF-7:5C and 10-fold in MCF-7:2A cells compared with MCF-7 cells and treatment with BZA significantly reduced the basal ERE activity in these cells (Fig. 3A). E2 treatment further increased ERE activity in MCF-7:5C and MCF-7:2A cells by 1.5- and 2.5-fold, respectively; however, in MCF-7 and T47D cells, the response was markedly more robust with a 12- and 20-fold increase, respectively (Fig. 3A).

To further test whether BZA is able to block ER α -regulated genes, we analyzed the expression level of pS2 mRNA in MCF-7:5C cells using quantitative RT-PCR. The pS2 gene is often used as a prognostic marker in breast cancer cells and is frequently used in studies of ER action. Furthermore, it is suggested that estrogen regulates the expression of pS2 through an imperfect ERE in the pS2 promoter (Berry et al., 1989). Our results showed that basal pS2 mRNA level was \sim 3.5-fold higher in MCF-7:5C cells compared with wild-type MCF-7 cells, and E2 treatment increased pS2 mRNA level by \sim 5.5-fold in MCF-7 cells and MCF-7:5C cells, which was

completely blocked by BZA (Fig. 3B). It is noteworthy that we also found that siRNA knockdown of ER α (Fig. 3C) significantly reduced the basal growth of MCF-7:5C cells and markedly reduced the inhibitory effect of BZA in these cells (Fig. 3C, bottom). In addition, suppression of ER α significantly reduced cyclin D1 protein in MCF-7:5C cells. Overall, these data indicate that in the absence of estrogen, the unliganded ER α drives the proliferation of hormone-independent breast cancer cells; however, in the presence of BZA, the ability to inhibit cell proliferation is dependent on receptor degradation.

BZA Blocks Cell Cycle Progression in MCF-7:5C Cells and Down-Regulates Cyclin D1. Because BZA significantly reduced the growth of MCF-7:5C cells, we next examined its effect on cell cycle progression. For experiment, MCF-7 and MCF-7:5C cells were treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 plus BZA for 48 h followed by propidium iodide staining and flow cytometric analysis. The results showed that in MCF-7:5C cells, E2 treatment significantly reduced the percentage of cells in S phase from 33 to 17% and marginally increased the percentage of cells in G₁ phase from 60 (control) to 66%, whereas BZA treatment increased the proportion of cells in the G₁ phase from 60 to 81%, and it reduced the proportion of S phase cells from 33 to 9% at 48 h. In MCF-7 cells, treatment with E2 increased the proportion of S phase cells from 19 to 42% at 48 h with no effect observed with BZA alone (Fig. 4A). It is noteworthy that the inhibitory

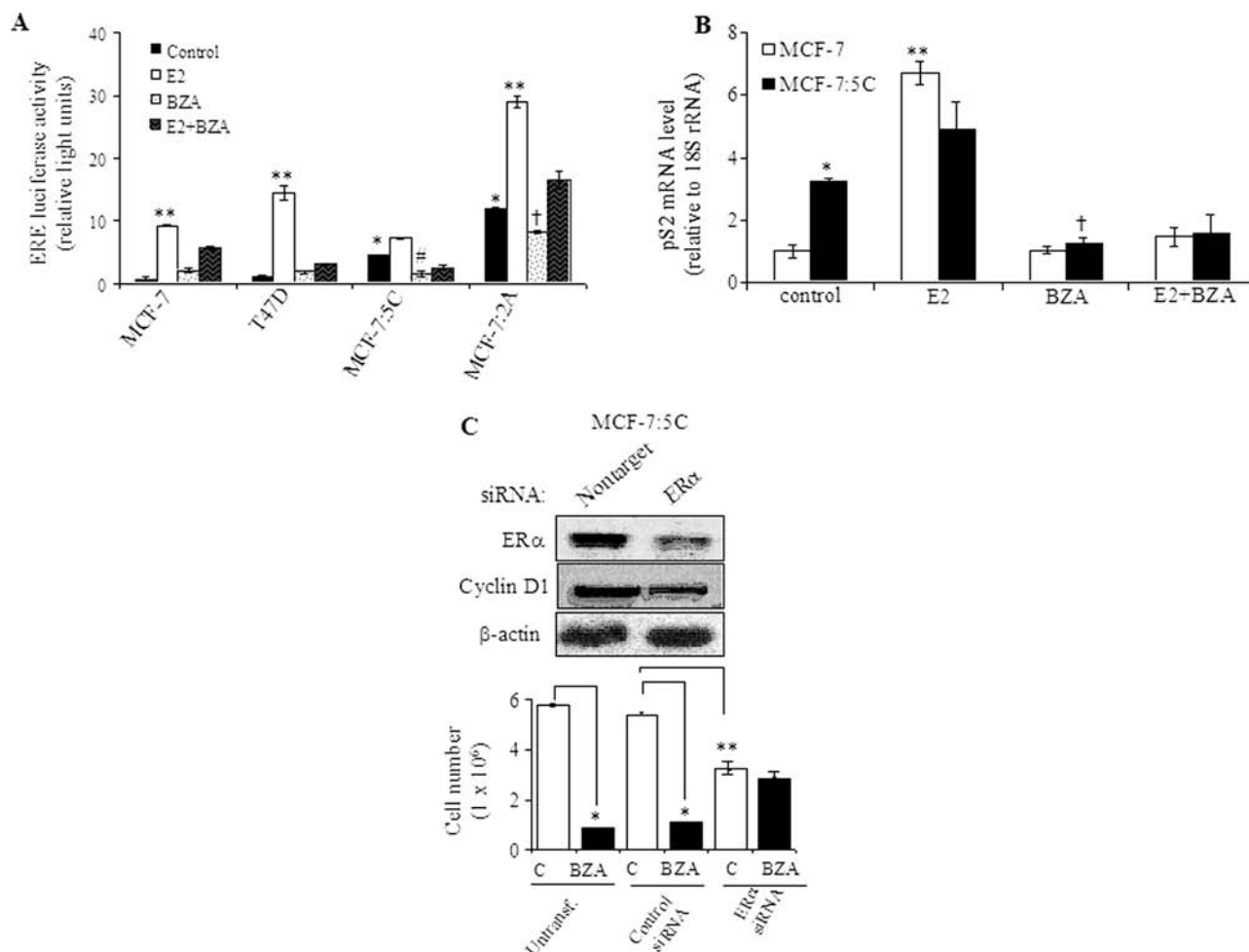


Fig. 3. BZA inhibits constitutive ER α transcriptional activity in hormone-independent and hormone-dependent breast cancer cells. **A**, ERE luciferase activity in hormone-dependent MCF-7 and T47D cells and hormone-independent MCF-7:5C and MCF-7:2A cells. For experiment, cells were transiently transfected with a 5 \times ERE-luciferase reporter construct and treated with 10^{-9} M E2, 10^{-7} M BZA, E2 + BZA, or nothing (control) for 24 h. Luciferase values for the treatment groups are reported as relative luciferase units. *, $p < 0.001$ compared with MCF-7 and T47D cells (control); **, $p < 0.0001$ compared with control for each cell line; #, $p < 0.01$ compared with untreated MCF-7:5C cells (control); †, $p < 0.05$ compared with untreated MCF-7:2A cells. **B**, real-time RT-PCR analysis of pS2 mRNA gene expression in MCF-7 and MCF-7:5C cells after treatments with E2 (10^{-9} M), BZA (10^{-7} M), or E2 + BZA for 24 h. Each data point represents the average of three biological replicates. *, $p < 0.01$ compared with untreated MCF-7 cells (control); **, $p < 0.001$ compared with untreated MCF-7 cells (control); †, $p < 0.001$ compared with untreated MCF-7:5C cells (control). **C**, MCF-7:5C cells were transfected with 100 nM nonspecific control or ER α siRNA for 48 h. Transfected cells were then harvested for Western blot analysis to detect ER α and cyclin D1 protein (top) or treated with 10^{-7} M BZA for an additional 4 days followed by cell counting using a hemocytometer (bottom). Data shown are representative of three independent experiments. *, $p < 0.001$ compared with untransfected control and nonspecific transfected cells; **, $p < 0.01$ compared with nonspecific transfected cells.

effect of BZA on cell cycle in MCF-7:5C cells was somewhat comparable with the pure antiestrogen fulvestrant; however, none of the other tested SERMs had any effect on cell cycle (data not shown).

Because BZA induced G₁-phase cell cycle block in MCF-7:5C cells, we further investigated the G₁-specific protein cyclin D1 in these cells. MCF-7 and MCF-7:5C cells were treated with BZA, E2, RAL, 4OHT, or FUL for 24 h, and lysates were prepared and analyzed by immunoblotting. Figure 4B shows that cyclin D1 was undetectable in untreated MCF-7 cells; however, treatment with E2 and, to a lesser extent, with 4OHT markedly increased cyclin D1 protein in these cells. In contrast, we found that cyclin D1 protein was constitutively overexpressed in MCF-7:5C and MCF-7:2A cells, and treatment with BZA completely reduced cyclin D1 protein in MCF-7:5C cells but not MCF-7:2A cells (Fig. 4B). It is noteworthy that none of the other SERMs inhibited cyclin D1 in MCF-7:5C cells; however, FUL significantly reduced

cyclin D1 protein level at 96 h, and it markedly reduced cyclin A protein in these cells (Supplemental Fig. 3). Time course experiments revealed that BZA inhibited basal cyclin D1 protein in a time-dependent manner with measurable effects observed as early as 2 h after treatment and maximum reduction at 24 h (Fig. 4C, top). BZA also reduced cyclin D1 mRNA (Fig. 4C, bottom) and cyclin D1 promoter activity (Fig. 4C, top right) in MCF-7:5C cells. Finally, we found that siRNA knockdown of cyclin D1 (Fig. 5A) significantly reduced the hormone-independent growth of MCF-7:5C cells (Fig. 5B), and it significantly reduced the ability of BZA to induce G₁ blockade in these cells (Fig. 5C), thus confirming the importance of cyclin D1 in the inhibitory action of BZA in these cells.

Molecular Modeling and Docking of BZA into the Ligand Binding Site of ER α . Molecular modeling and docking studies were carried out in an attempt to predict the bioactive conformation of BZA and to understand the molec-

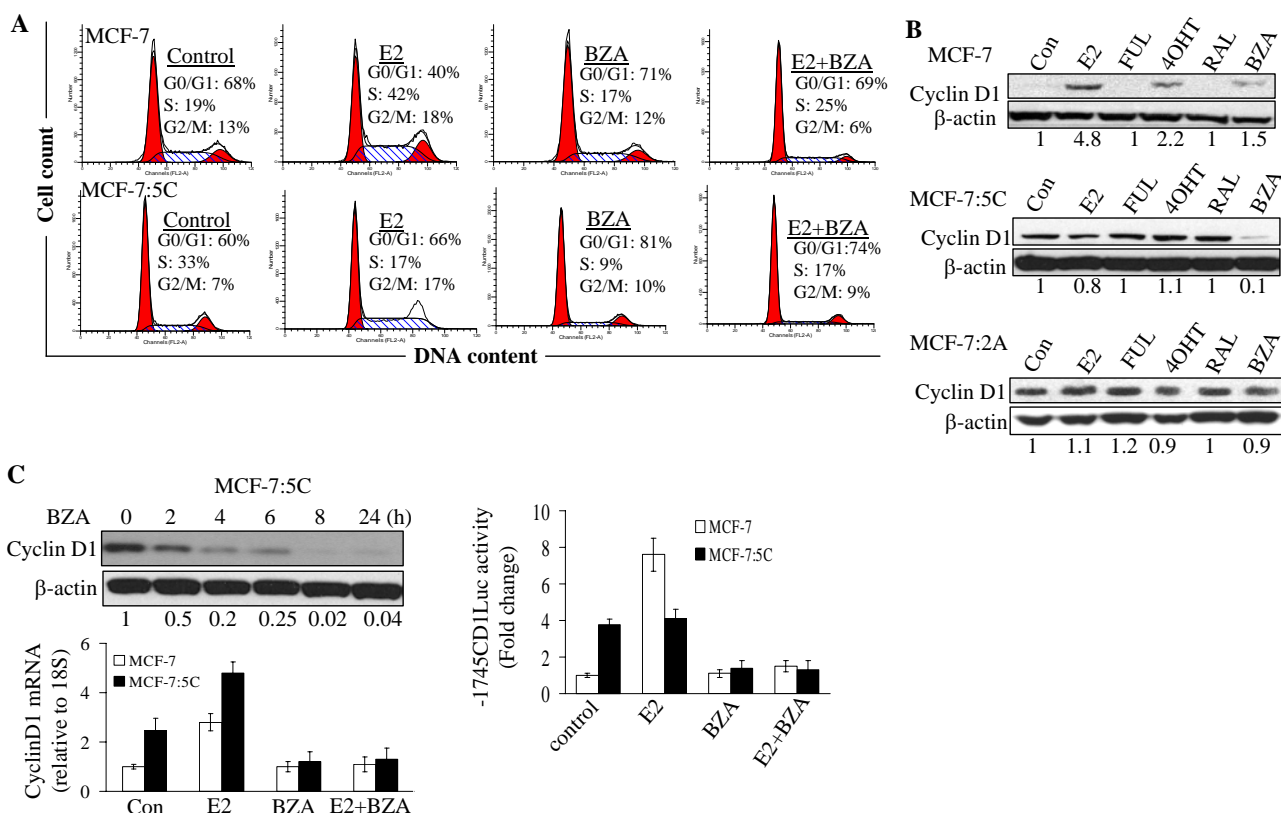


Fig. 4. Effects of BZA on cell cycle progression and cyclin D1 regulation in MCF-7 and MCF-7:5C cells. **A**, cell cycle distribution was determined by propidium iodide staining of DNA content and flow cytometry. Cells were treated with 10^{-9} M E2, 10^{-7} M BZA, or E2 plus BZA for 24 and 48 h. Thirty thousand cells per sample and three replicates per group were collected. Representative histograms are shown. **B**, Western blot analysis of cyclin D1 expression level in MCF-7 and MCF-7:5C cells after treatment with BZA and other SERMs. Before the experiment, MCF-7 cells were switched from fully estrogenized media to estrogen-free media for 3 days and then treated with ethanol vehicle (control), 10^{-9} M E2 alone, or 10^{-9} M E2 plus FUL (10^{-7} M), RAL (10^{-7} M), 4OHT (10^{-7} M), or BZA (10^{-7} M) for 24 h. MCF-7:5C cells, however, did not require a media switch because they are hormone-independent and are routinely grown in estrogen-free media. MCF-7:5C cells were treated as described above for MCF-7 cells. Quantitated protein levels normalized to β -actin are indicated. **C**, BZA regulation of cyclin D expression and promoter activity in MCF-7:5C cells. Cells were treated with 10^{-7} M BZA for the indicated time points. Cyclin D1 protein and mRNA levels were determined by Western blot and quantitative RT-PCR, respectively, with β -actin and 18S rRNA as internal controls. For cyclin D1 promoter activity experiment, MCF-7 and MCF-7:5C cells were cotransfected with a full-length cyclin D1 promoter plasmid (-1745 CD1Luc) and *Renilla reniformis* luciferase control plasmid overnight and then treated with 10^{-9} M E2, 10^{-8} M BZA, or E2 + BZA for 24 h. Luciferase activity was measured as described under *Materials and Methods*. Each point represents the mean of three determinations \pm S.E.M.

ular basis of interaction of this ligand with ER α . Using the available X-ray crystallographic data, the flexible docking of BZA into the ligand binding domain of ER α cocrystallized with RAL was performed, and for comparison reasons, FUL and RAL were also docked in their native protein structure. The superimposition of the docked solution and experimental structure of RAL shows that the docking model recapitulates the orientation of the native ligand in the active site, and the same interactions with the key amino acids of the binding cavity are formed with a ligand root mean square deviation of 0.362 compared with the crystal structure (Fig. 6A). The experimental structure of ER α cocrystallized with E2 (PDB code [1gwr](#)), the agonist conformation of the receptor, is displayed in Fig. 6B, whereas the experimental antagonist conformation of ER α bound to 4OHT and RAL are superimposed and presented in Fig. 6C. The docking results analysis reveals that BZA binds to ER α in an antagonist orientation similar with RAL (Fig. 6D) and has the tendency to form the same hydrophobic contacts with the amino acids lining the binding cavity. In addition, the same complex H-bond network is formed with Asp351, Glu353, Arg394, His524, and a highly ordered water molecule, located in the vicinity of residues Glu353 and Arg394

(Fig. 6D). However, we should note that a number of residues adopt different conformations in the Induced Fit Docking (IFD) poses compared with the experimental structure of ER α , PDB code [1err](#) (Supplemental Fig. 4). The most significant difference has been observed for Leu539 of helix 12. The larger ring of BZA causes the side chain of Leu539 to be pushed away from its original position by approximately 1 Å. In all top-ranked IFD structures (four poses having the composite score of 0.5 kcal/mol), Leu529 side chain is moved up from its original orientation toward the ring of BZA to optimize the hydrophobic contacts between the ligand and residue side chain (Supplemental Fig. 4). We also compared the docked structure of BZA with the binding mode of 4OHT to ER α (Fig. 6C) and superimposed it in the binding site of 4OHT-ER α complex (Fig. 6E). The 4OHT bound receptor shows that the H-bond between BZA and H524 is missing (Fig. 6E) because of the different orientation of this amino acid in the binding site compared with the RAL-ER α complex (Fig. 6C). When FUL was docked to RAL-ER α complex (Fig. 7A), the H-bond network was recapitulated with one exception: the interaction with Asp351 is missing, whereas the flexible side chain of FUL fills the groove between helix 3 and helix 12 (Fig. 7B).

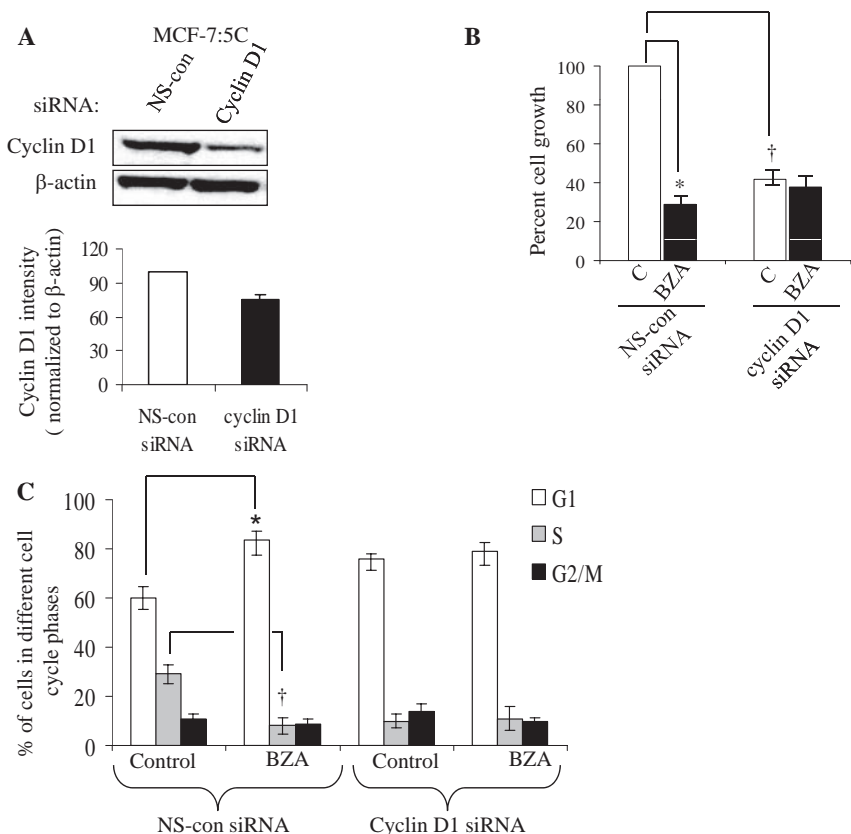


Fig. 5. Effect of cyclin D1 knockdown on proliferation and cell cycle in MCF-7:5C cells. **A**, Western blot analysis of cyclin D1 protein expression in MCF-7:5C cells transfected with 100 nM cyclin D1 siRNA or the nonspecific (NS)-control siRNA, as determined 72 h after transfection. **B**, cell growth of transfected cells treated with 100 nM BZA or vehicle (control). Transfected cells (30,000/well) were seeded in 24-well dishes overnight and then treated with BZA for 5 days. After treatment, cells were collected and counted using a hemocytometer. Data is presented as percentage and is based on the mean from three independent experiments with duplicate (*, $p < 0.01$ versus nontarget transfected cells). **C**, cell cycle analysis of cyclin D1 siRNA-transfected and control siRNA transfected MCF-7:5C cells after treatment with BZA for 48 h. Data are based on the mean from three independent experiments with duplicate. *, $p < 0.01$; **, $p < 0.001$.

Overall, these findings indicate that the alignment of BZA in the binding pocket of ER α predicted by the IFD is similar with that predicted via the rigid docking method (Glide) and with the alignment of RAL in the experimental structure, PDB code [1err](#). However, there are a few differences in the orientation of some residues in the binding site when the docking of BZA is performed with IFD protocol, and these differences might help to explain the different biological effects of BZA versus RAL in our cell model.

Discussion

In the present study, we report for the first time that BZA inhibits the growth of breast cancer cells that have acquired resistance to long-term estrogen deprivation (i.e., hormone-independent/aromatase inhibitor resistant). Specifically, we found that BZA at 10^{-8} M inhibited the growth of hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells by 80 and 55%, respectively. The inhibitory effect of BZA in MCF-7:5C cells was associated with G₁ arrest and cyclin D1 and ER α down-regulation, whereas in MCF-7:2A cells, BZA suppressed cyclin A with marginal effects on cyclin D1. The pure antiestrogen FUL also inhibited the growth of MCF-7:5C cells by inducing G₁ arrest; however, it did not down-regulate cyclin D1 until 96 h, which was 48 h after its effect on cell cycle. Strikingly, RAL, 4OHT, and ENDOX failed to inhibit cyclin D1 expression in MCF-7:5C cells, and these compounds did not have any growth-inhibitory effect in MCF-7:5C cells. Although it is not entirely clear why BZA was more potent than fulvestrant at inhibiting the growth of MCF-7:5C cells, one possibility might be due to the fact that BZA down-regulated both ER α and cyclin D1, whereas FUL down-regulated ER α and had marginal effects on cyclin D1,

which was observed at 96 h. Molecular modeling studies indicated that BZA bound the ligand binding domain of ER α in an antagonist orientation similar to RAL (Fig. 6D) but distinct from 4OHT (Fig. 6E) and fulvestrant (Fig. 7). However, a few differences were noticed in the orientation of some residues in the binding site when the docking of BZA was performed with the IFD protocol. The most significant difference was observed for the Leu539 of helix 12. The larger ring of BZA caused the side chain of Leu539 to be pushed away from its original position by approximately 1 Å. This alteration in the orientation of Leu539 side chain could trigger a conformational change of helix 12, which in turn could lead to the recruitment of other proteins by the BZA-ER α compared with the RAL-ER α complex. Indeed, these findings help to further distinguish BZA from the other SERMs such as TAM and RAL, and they support the concept that subtle but moderate structural differentiation can dramatically affect the ability of a ligand to regulate cell proliferation.

Previous research has indicated that deregulation of ER α expression is a driving force in the initiation and progression of estrogen-sensitive breast tumors (Garcia-Closas and Chacko, 2008; Garcia-Closas et al., 2008). It has been suggested that alterations in pathways leading to ER α synthesis and/or degradation underlie the deregulation of ER α and its consequent manifestations, including enhanced proliferation in breast tumors (Sommer and Fuqua, 2001). ER α is the predominant receptor isoform expressed in breast cancer cells, and increased numbers of ER α -expressing cells can be observed at the earliest stages of breast tumorigenesis. We have shown previously that ER α mRNA and protein levels are significantly elevated in breast cancer cells that have been adapted to grow in an estrogen-depleted environment

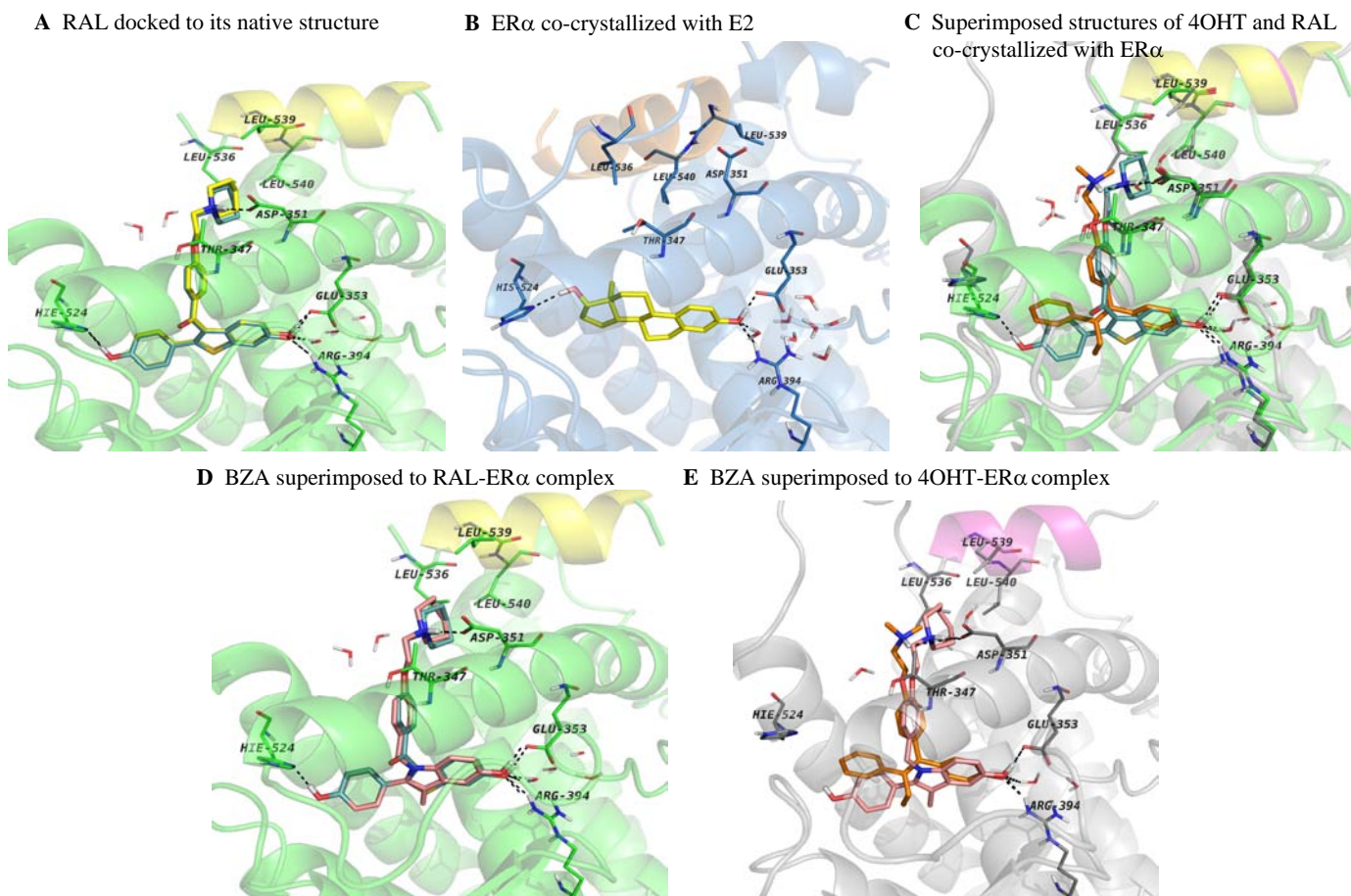


Fig. 6. Molecular modeling of ER α binding site with various ligands. A, comparison between the experimental (yellow sticks) and top ranked docking pose (cyan sticks) of RAL to ER α binding site. The docking pose recapitulates very well the alignment of the cocrystallized ligand in the receptor binding site having a ligand root mean square deviation of 0.36 Å. B, agonist conformation of ER α cocrystallized with E2; helix 12 is depicted in orange and lays over the binding site sealing the ligand inside it. The antagonist conformations of the receptor are shown in C, D, and E. X-ray structures of ER α cocrystallized with 4OHT (C), raloxifene (D), and bazedoxifene (E) docked into the ER α -raloxifene crystal structure. Helix 12 is depicted in magenta for 4OHT bound conformation and yellow for raloxifene and bazedoxifene. In addition, the key amino acids lining the binding site are displayed and the network of hydrogen bonds in which they are involved with the ligands is shown in black dashed lines. Carbon atoms are colored in yellow for E2, orange for 4OHT, cyan for raloxifene, and pink for bazedoxifene. These images show the differences between the agonist (B) and antagonist conformation (C, D, and E) of ER α and present the alignment of bazedoxifene in the binding site of ER α , which is similar to raloxifene's orientation, and the same interactions with the key amino acids of the binding cavity are encountered.

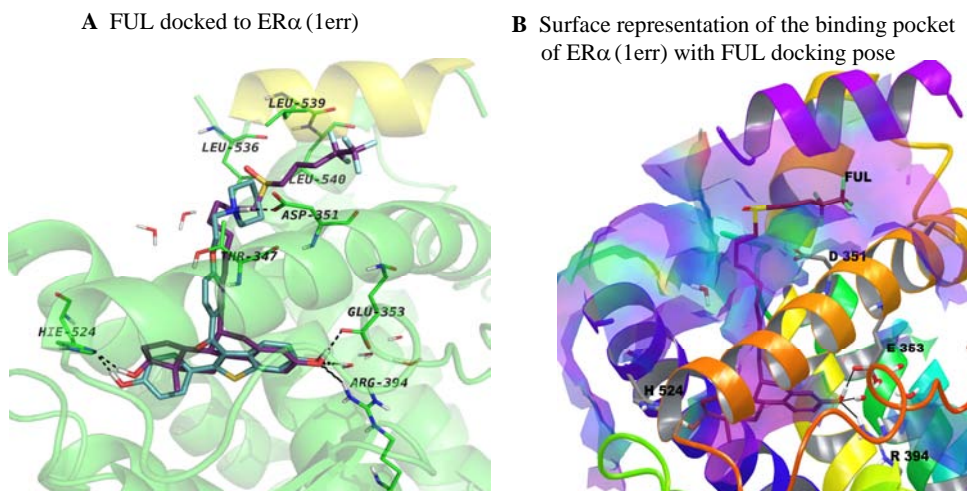


Fig. 7. Simplified representations of the ER α binding site with fulvestrant. A, representation of the ER α binding site with the best docking pose for fulvestrant (FUL, purple sticks). B, surface representation of ER α binding site accommodating FUL. Hydrophobic areas are mapped in purple, whereas the hydrophilic parts are colored in light yellow-green. The binding site accommodates very well the ligand, which forms the H-bond contacts with the same amino acids like E2 or RAL, whereas the aliphatic side chain protrudes from the binding site and lies in the groove between helix 3 (orange cartoon) and helix 12 (purple cartoon). Only the key amino acids underlying the binding site are shown.

(Murphy et al., 1990; Pink et al., 1996; Lewis et al., 2005a). This particular type of regulation in which ER α levels are increased after estrogen deprivation has been described as a model I response (Pink and Jordan, 1996). A model I re-

sponse is characterized by an ER α that is expressed at high levels in the absence of estrogen and is subsequently down-regulated after estrogen binding, primarily through repression of the steady-state level of the mRNA. In the present

study, we found that basal ER α protein levels were up-regulated greater than 3-fold in hormone-independent MCF-7:5C and MCF-7:2A breast cancer cells compared with MCF-7 and T47D cells, and treatment with BZA (10^{-8} M) induced proteasome-mediated degradation of ER α in these cells, which was reversed by the proteasome inhibitor MG132. The ability of BZA to degrade ER α in MCF-7:5C cells was rapid and robust, occurring as early as 4 h after treatment with maximum degradation at 24 h. It is noteworthy that BZA and fulvestrant were the only compounds that markedly reduced the growth of both MCF-7:5C and MCF-7:2A breast cancer cells, and blocking BZA-induced ER α degradation with MG132 dramatically reduced its growth inhibitory effects on these cells (data not shown). The importance of ER α in mediating the antagonist effects of BZA in hormone-independent MCF-7:5C cells was further confirmed by siRNA knockdown experiments, which showed a 60% reduction in the ability of BZA to inhibit the growth of these cells. Suppression of ER α also significantly reduced the basal growth of MCF-7:5C cells and E2-induced growth in wild-type MCF-7 cells, which is consistent with recent findings by Ariazi et al. (2010). It should be noted, however, that degradation or suppression of ER α is not the only mechanism by which an antagonist can inhibit cell proliferation. For example, TAM has been shown to stabilize ER α protein against degradation in breast cancer cells (Murphy et al., 1990; Pink et al., 1995, 1996; Pink and Jordan, 1996); however, it is a potent antagonist in the breast with the ability to block E2-stimulated proliferation and E2-induced ERE activity in these cells.

Apart from ER α , BZA also significantly reduced cyclin D1 expression in hormone-independent MCF-7:5C breast cancer cells. Cyclin D1 is a breast cancer oncogene whose overexpression has been linked to poor prognosis in ER α and progesterone receptor-positive breast cancers (Lammie and Peters, 1991). It is a multifunctional G₁-phase cyclin whose regulatory effects are particularly important in breast development and cancer (Sutherland and Musgrove, 2004). Cyclin D1 is highly induced by estrogen (Said et al., 1997), and it contributes to poor treatment response of ER-positive tumors by acting downstream to promote hormone agonist- and antagonist-independent proliferation (Wilcken et al., 1997). We found that cyclin D1 protein was constitutively elevated by 3- to 5-fold in hormone-independent MCF-7:5C and MCF-7:2A cells compared with wild-type MCF-7 and T47D cells, and treatment with BZA reduced it to an undetectable level in MCF-7:5C cells but not MCF-7:2A cells. In addition, we found that suppression of cyclin D1 in MCF-7:5C cells reduced the hormone-independent growth of these cells, and it significantly reduced the ability of BZA to inhibit cell growth and induce cell cycle arrest in these cells. Suppression of cyclin D1 also significantly reduced ER α protein levels in MCF-7:5C cells with similar effects observed after ER α suppression, thus suggesting a link between cyclin D1 and ER α in these cells. Indeed, a connection between ER and cyclin D1 was demonstrated previously when cyclin D1 was shown to interact directly with the ligand-binding domain of ER and stimulate ER transactivation in a ligand-independent fashion (Zwijsen et al., 1997). More recently, cyclin D1 was shown to interact with coactivators of the SRC-1 family through a motif that resembles the leucine-rich coactivator binding motif of nuclear receptors. By acting as a bridging factor be-

tween ER and SRCs, it is believed that cyclin D1 can recruit SRC family coactivators to ER in the absence of ligand. It is worth noting that hormone-independent MCF-7:5C cells express elevated levels of SRC-1 protein compared with hormone-dependent MCF-7 cells, and BZA treatment significantly reduces basal SRC-1 levels in these cells (data not shown).

Although cyclin D1 gene transcription is directly induced by estrogen, there is no estrogen response element in it. Instead, the cyclin D1 promoter contains multiple regulatory elements, including binding sites for activator protein-1, signal transducer and activator of transcription 5, nuclear factor- κ B, cAMP response element, SP1, and E2F. A fragment between -994 and -136 of the cyclin D1 promoter was shown previously to be estrogen-responsive, and this region has binding sites for AP-1 and SP-1 (Altucci et al., 1996). We have reported that estrogen-induced cyclin D1 transactivation in MCF-7 breast cancer cells was mediated by the CRE region, which is known to bind activating transcription factor 2 (Lewis et al., 2005c,d). A notable finding of our study was that basal cyclin D1 promoter activity was significantly elevated in hormone-independent MCF-7:5C cells compared with hormone-dependent MCF-7 cells and treatment with BZA completely reduced the promoter activity in these cells to the level seen in the untreated MCF-7 cells. In contrast, E2 did not induce cyclin D1 expression or promoter activity in hormone-independent MCF-7:5C cells, whereas in hormone-dependent MCF-7 cells, it increased cyclin D1 protein level by 3-fold and its promoter activity by 4-fold, which is consistent with its function as a proapoptotic agent in MCF-7:5C cells versus an agonist in MCF-7 cells.

In conclusion, it is clear from clinical data that BZA in combination with conjugated estrogens represents a new form of therapeutic agents for the treatment of postmenopausal symptoms and prevention of postmenopausal osteoporosis. The fact that it does not stimulate the breast or endometrium and is very effective at inhibiting the proliferation of endocrine-resistant breast cancer cells highlights its widespread therapeutic potential and demonstrates that not all SERMs are alike. Our data also suggest that the overexpression of ER α and cyclin D1 in MCF-7:5C cells might be driving the hormone-independent growth of these cells and that the ability of BZA to down-regulate ER α and cyclin D1 is critical to treat and possibly reverse antihormone resistance in breast cancer.

Acknowledgments

We thank Dr. James Ingle for the endoxifen compound and Drs. Richard Pestell and Chris Albanese for the full-length cyclin D1 promoter plasmid.

Authorship Contributions

Participated in research design: Lewis-Wambi and Jordan.
Conducted experiments: Lewis-Wambi and Kim.
Contributed new reagents or analytic tools: Curpan, Grigg, and Sarker.
Performed data analysis: Lewis-Wambi.
Wrote or contributed to the writing of the manuscript: Lewis-Wambi and Jordan.

References

Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, and Pestell RG (1995) Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* 270:23589–23597.

- Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, and Weisz A (1996) 17 β -Estradiol induces cyclin D1 gene transcription, p36D1–p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene* **12**: 2315–2324.
- Archer DF, Pinkerton JV, Utian WH, Menegoci JC, de Villiers TJ, Yuen CK, Levine AB, Chines AA, and Constantine GD (2009) Bazedoxifene, a selective estrogen receptor modulator: effects on the endometrium, ovaries, and breast from a randomized controlled trial in osteoporotic postmenopausal women. *Menopause* **16**: 1109–1115.
- Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI, et al. (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* **70**:1184–1194.
- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, and Bourne PE (2000) The Protein Data Bank. *Nucleic Acids Res* **28**:235–242.
- Berry M, Nunez AM, and Chambon P (1989) Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. *Proc Natl Acad Sci USA* **86**:1218–1222.
- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA, and Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**:753–758.
- Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, et al. (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* **47**:1739–1749.
- Garcia-Closas M and Chanock S (2008) Genetic susceptibility loci for breast cancer by estrogen receptor status. *Clin Cancer Res* **14**:8000–8009.
- Garcia-Closas M, Hall P, Nevanlinna H, Pooley K, Morrison J, Richesson DA, Bojesen SE, Nordestgaard BG, Axelsson CK, Arias JI, et al. (2008) Heterogeneity of breast cancer associations with five susceptibility loci by clinical and pathological characteristics. *PLoS Genet* **4**:e1000054.
- Guallar V, Jacobson M, McDermott A, and Friesner RA (2004) Computational modeling of the catalytic reaction in triosephosphate isomerase. *J Mol Biol* **337**: 227–239.
- Jiang SY and Jordan VC (1992) Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J Natl Cancer Inst* **84**:580–591.
- Jiang SY, Wolf DM, Yingling JM, Chang C, and Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* **90**:77–86.
- Jordan VC (2007) New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* **72**:829–842.
- Jordan VC (2008) The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer—survival or death? *J Clin Oncol* **26**:3073–3082.
- Jordan VC (2009) A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* **69**:1243–1254.
- Kagan R, Williams RS, Pan K, Mirkin S, and Pickar JH (2010) A randomized, placebo- and active-controlled trial of bazedoxifene/conjugated estrogens for treatment of moderate to severe vulvar/vaginal atrophy in postmenopausal women. *Menopause* **17**:281–289.
- Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, and Lyttle CR (2005) Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology* **146**:3999–4008.
- Labarca C and Paigen K (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* **102**:344–352.
- Lammie GA and Peters G (1991) Chromosome 11q13 abnormalities in human cancer. *Cancer Cells* **3**:413–420.
- Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, and Jordan VC (2008a) Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. *Eur J Cancer* **44**:1770–1779.
- Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle JR, Klein-Szanto AJ, and Jordan VC (2008b) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* **10**:R104.
- Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, and Jordan VC (2005a) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* **97**:1746–1759.
- Lewis JS, Osipo C, Meeke K, and Jordan VC (2005b) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* **94**:131–141.
- Lewis JS, Thomas TJ, Pestell RG, Albanese C, Gallo MA, and Thomas T (2005c) Differential effects of 16 α -hydroxyestrone and 2-methoxyestradiol on cyclin D1 involving the transcription factor ATF-2 in MCF-7 breast cancer cells. *J Mol Endocrinol* **34**:91–105.
- Lewis JS, Vijayanathan V, Thomas TJ, Pestell RG, Albanese C, Gallo MA, and Thomas T (2005d) Activation of cyclin D1 by estradiol and spermine in MCF-7 breast cancer cells: a mechanism involving the p38 MAP kinase and phosphorylation of ATF-2. *Oncol Res* **15**:113–128.
- Maximov PY, Myers CB, Curpan RF, Lewis-Wambi JS, and Jordan VC (2010) Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* **53**:3273–3283.
- Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, Moran RA, Henderson RA, Bender RH, Unwalla RJ, et al. (2001) Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem* **44**:1654–1657.
- Miller PD, Chines AA, Christiansen C, Hoeck HC, Kendler DL, Lewiecki EM, Woodson G, Levine AB, Constantine G, and Delmas PD (2008) Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. *J Bone Miner Res* **23**:525–535.
- Murphy CS, Pink JJ, and Jordan VC (1990) Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Res* **50**:7285–7292.
- Pink JJ, Bilimoria MM, Assikis J, and Jordan VC (1996) Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* **74**:1227–1236.
- Pink JJ, Jiang SY, Fritsch M, and Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* **55**:2583–2590.
- Pink JJ and Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* **56**:2321–2330.
- Pinkerton JV, Archer DF, Utian WH, Menegoci JC, Levine AB, Chines AA, and Constantine GD (2009) Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. *Menopause* **16**:1102–1108.
- Said TK, Conneely OM, Medina D, O'Malley BW, and Lydon JP (1997) Progesterone, in addition to estrogen, induces cyclin D1 expression in the murine mammary epithelial cell, in vivo. *Endocrinology* **138**:3933–3939.
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, and Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**:927–937.
- Silverman SL, Christiansen C, Genant HK, Vukicevic S, Zanchetta JR, de Villiers TJ, Constantine GD, and Chines AA (2008) Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. *J Bone Miner Res* **23**:1923–1934.
- Sommer S and Fuqua SA (2001) Estrogen receptor and breast cancer. *Semin Cancer Biol* **11**:339–352.
- Sutherland RL and Musgrove EA (2004) Cyclins and breast cancer. *J Mammary Gland Biol Neoplasia* **9**:95–104.
- Wärnmark A, Treuter E, Gustafsson JA, Hubbard RE, Brzozowski AM, and Pike AC (2002) Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor α . *J Biol Chem* **277**:21862–21868.
- Wilcken NR, Prall OW, Musgrove EA, and Sutherland RL (1997) Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. *Clin Cancer Res* **3**:849–854.
- Wu X, Hawse JR, Subramaniam M, Goetz MP, Ingle JN, and Spelsberg TC (2009) The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor α for degradation in breast cancer cells. *Cancer Res* **69**:1722–1727.
- Zwijnen RM, Wientjens E, Klompmaaker R, van der Sman J, Bernards R, and Michalides RJ (1997) CDK-independent activation of estrogen receptor by cyclin D1. *Cell* **88**:405–415.

Address correspondence to: Dr. Joan Lewis-Wambi, Women's Cancer Program, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. E-mail: joan.lewis@fccc.edu

Profile of V. Craig Jordan

In the mid-1970s, breast cancer survival rates were dismal. Researchers hoped to find a drug capable of thwarting the disease, but the prospects were few and far between. In a laboratory on the campus of the Worcester Foundation for Experimental Biology in Shrewsbury, Massachusetts, a group of experimental rats were dying from breast cancer. A researcher gave them a triphenyl ethylene—a purported antiestrogen—with the slim hope that it would slow progression of the disease. The cancer disappeared (1). Within a few years, a clinical trial of the drug was launched among women suffering from breast cancer. The women's tumors, just as those in the rats, shrank. By 1978, the US Food and Drug Administration had approved a triphenyl ethylene-based drug known as tamoxifen for the treatment of late-stage breast cancer (2).

Today, tamoxifen is a resounding success. By the numbers, breast cancer mortality rates held steady from 1975 to 1990 but declined by almost 20% from 1990 to 2000. Two-thirds of that decline is attributable to adding tamoxifen to the chemotherapy regimen already used to treat breast cancer. Among breast cancer survivors taking tamoxifen for 5 years, the standard dosage for the drug, mortality declined by nearly 40% (3, 4). The researcher who cured the rats, V. Craig Jordan, is now known as the "Father of Tamoxifen."

Since discovering tamoxifen's potential to prevent breast cancer more than three decades ago, Jordan, a 2009 inductee to the National Academy of Sciences, has devoted his career to understanding the characteristics of the drug—its benefits, pitfalls, and other applications. Thanks to that work, it is now known that tamoxifen and similar drugs act as both estrogen inhibitors and estrogens, depending on where they travel inside the body. Collectively, the drugs are referred to as selective estrogen receptor modulators, or SERMs. SERMs are now routinely prescribed to treat not just breast cancer but other estrogenic disorders, such as osteoporosis. Jordan says there is hope of someday using this same class of drugs to reduce the devastation of coronary heart disease.

In his Inaugural Article, Jordan returns to the topic of breast cancer to explain a paradox in the literature that has plagued scientists for decades. From his own work, Jordan knew that tamoxifen's antiestrogenic properties stopped the growth of breast cancer. However, in the 1940s, another researcher by the name of Alexander Haddow showed that giving postmenopausal women estrogen also caused the disease to grind to a halt (5). Now



V. Craig Jordan.

Jordan has explained how estrogen can both promote and prevent breast cancer. "We have solved a 70-year mystery," he says (6).

Early Childhood

Jordan, the Alfred G. Knudson Jr. Chair in Cancer Research at the Fox Chase Cancer Center in Philadelphia, Pennsylvania, was born in New Braunfels, Texas, in 1947 but moved to the United Kingdom as an infant with his British parents. Growing up, Jordan developed a deep, almost singular, infatuation with chemistry—the origins of which he cannot recall. At age 13, his mother let him build a chemistry laboratory in his bedroom, a prescient if costly decision.

"There were always fires in the bedroom and bombs going off in the back of the garden," Jordan says, recounting an experiment with sodium chlorate that went horribly awry. Rather than blow up the house, Jordan chucked the whole, smoldering mess out the window—creating a crater-sized gap where grass once grew. "My parents were furious," Jordan says. Telling them not to worry, Jordan reseeded the lawn and added some copper sulfate to expedite the growing process. "The grass did grow back," Jordan says, "but it was blue."

However, where Jordan excelled in chemistry, he floundered in other "lesser" subjects. "I thought plants were stupid," he says. By age 16, when he needed to pass five subject examinations to continue his education, Jordan only passed three, forcing his mother to beg the headmaster

to let him retake the tests in a few months. Luckily, he passed.

By then, Jordan had become a tutor to his peers, teaching them the basics of chemistry, pharmacology, and biochemistry. Seeing that talent, a teacher by the name of Charles Bescoby convinced Jordan and his parents that he should not go to work as a technician at nearby Imperial Chemical Industries (ICI) Pharmaceuticals as he had long planned, but to university. Jordan received admission to the University of Leeds and graduated with a degree in pharmacology in 1969. He stayed on for another 3 years to receive his doctorate in the same subject, by then convinced that his future lay in developing a drug to treat cancer—a monumental chemistry challenge that appealed to Jordan's intellect.

ICI 46,474

However, Jordan's path to becoming a cancer drug expert was roundabout. At Leeds, he had extensively studied triphenyl ethylenes, the active compound in a drug that ICI had once believed would become the world's first-ever "morning-after pill" (7).

Going by the code name ICI 46,474, the drug had been shown to block estrogen from reaching the uterus in rats. However, hopes were dashed when a clinical trial in humans found that more women got pregnant when taking the drug than not (8). Jordan was studying to see just how that drug worked in the body—a complex, voluminous project. When he went to defend his thesis in 1972, the university had no experts on staff capable of grasping Jordan's thesis. So they called in Arthur Walpole, a researcher at ICI. Walpole held the patent on ICI 46,474 and was thus well placed to make sense of Jordan's opus.

After that chance encounter, Walpole helped Jordan line up a postdoctoral fellowship at the Worcester Foundation for Experimental Biology. He was to work with endocrinologist Michael Harper to develop new contraception methods. By the time Jordan arrived in Massachusetts, though, Harper had accepted another job, and the Worcester Foundation told Jordan to set up his own laboratory for 2 years. "I was on my own," Jordan says, with no idea of what to research. So he called Walpole, and the two men discussed turning ICI 46,474 into a drug to treat breast cancer.

This is a Profile of a recently elected member of the National Academy of Sciences to accompany the member's Inaugural Article on page 18879.

Despite the failure of ICI 46,474 as a morning-after pill, Walpole and Jordan knew that the drug had antiestrogenic properties. Although breast cancer has different causes, for most women it arises when estrogen binds to receptor sites in breast cancer cells, allowing them to proliferate. A drug capable of binding to and inactivating those receptors might just thwart the spread of the disease, Walpole theorized. As a contraception researcher, Walpole had no opportunity to research that idea. So he handed the project over to Jordan.

In the early 1970s, Jordan induced rats to develop mammary (breast) cancer and confirmed that the tumors needed estrogen hormones to survive. When the rats were given ICI 46,474 the tumors shrank—a situation only mirrored in rats whose ovaries had been removed (1). ICI 46,474, he concluded, held promise as a drug to treat and prevent breast cancer in women with estrogen receptor sites in their breast cancer cells.

The idea that a drug could prevent breast cancer, however, remained controversial. Jordan's paper was initially rejected before being accepted by the *European Journal of Cancer Research* in 1976. By then Jordan had completed his postdoctoral work in Massachusetts and become a full-time lecturer in pharmacology at his alma mater, the University of Leeds.

At Leeds, Jordan began studying how long tamoxifen should be administered in women with breast cancer. Using a rat tumor model, he showed that treatments shorter than a few years ultimately failed and the rats went on to develop tumors, whereas administering tamoxifen for longer periods thwarted the progression of the disease (9, 10). Today, the standard tamoxifen treatment extends over 5 years (11). Jordan's research eventually prompted ICI to launch clinical trials into the use of tamoxifen as drug to treat breast cancer. "Tamoxifen slowly became hot," Jordan says.

Two Faces of Tamoxifen

With tamoxifen poised for widespread rollout, however, Jordan began to worry that long-term estrogen deprivation through tamoxifen might trigger unforeseen side effects. Estrogen, he explains, is a double-edged sword for women. Although implicated in breast cancer, the hormone is also critical for the development of the cardiovascular system and bones. Jordan wondered whether long-term estrogen deprivation would lead to osteoporosis or heart disease. In 1980, he relocated to the University of Wisconsin, Madison, and started his own laboratory to research the health implications of using tamoxifen long term.

After finding that long-term tamoxifen use actually seemed to lessen the incidence of osteoporosis and heart disease in rodents (12), Jordan and colleagues launched a 2-year study of 140 postmenopausal women with a history of breast cancer. Half the women were treated with tamoxifen, whereas the other half received a placebo. As with the rodents, the researchers found that tamoxifen lowered cholesterol in women receiving the drug after 3 months and that such positive effects persisted for years (13). Similarly, bone density increased in women receiving tamoxifen but decreased in women receiving placebo (14).

Collectively, Jordan's research suggested that tamoxifen and another related drug, raloxifene, were not antiestrogenic everywhere in the body as previously assumed, but were selective estrogens and antiestrogens (SERMs). "It turns out that different tissues interpret the drugs' signal in different ways," Jordan says. "So, paradoxically, tamoxifen and raloxifene built bones."

Raloxifene is now widely prescribed to postmenopausal women in danger of developing osteoporosis (15). Estimates suggest that raloxifene use has inadvertently protected thousands of female users from developing breast cancer (16). The fact that women taking the drug report a lower incidence of breast cancer than the general population is just a "beneficial side effect," Jordan says (17).

Not all side effects of SERMs were desirable, however. In 1988, Jordan, working with then graduate student Marco Gottardis, showed that tamoxifen promoted the growth of endometrial tumors in women (18). However, subsequent research made clear that the benefits of using tamoxifen for the treatment of breast cancer far outweighed the risk of developing endometrial cancer. Today, tamoxifen is estimated to save approximately 30 times more women than it harms (19).

Interestingly, raloxifene did not promote the development of endometrial cancer, suggesting that it may be preferable to tamoxifen. However, *Cancer Prevention Results* published a 2010 update of a five-year study comparing the long-term health outcomes of women receiving tamoxifen with women receiving raloxifene. Although participants in both groups had equal outcomes after 41 months of treatment, tamoxifen emerged as the more effective weapon against the recurrence of breast cancer when that time frame doubled. Specifically, raloxifene was shown to be less than 80% as effective as tamoxifen (20, 21).

Estrogen as Cancer Killer

Despite all his headway into revealing tamoxifen's secrets, an issue that nig-

gled at Jordan throughout his career has been that of resistance. If tamoxifen required 5 years to adequately treat breast cancer, would that give cancer cells too much time to find a new way to undermine the drug?

As early as the mid-1980s, Gottardis developed a tamoxifen-resistant human tumor in mice. He further showed that such tumors could be transplanted into future generations of mice and kept alive with tamoxifen treatment (22). Long-term tamoxifen use in humans, Jordan says, "seemed like a recipe for disaster."

Critical to Jordan's thinking, however, was the belief that estrogen blockers are required to thwart the growth of breast cancer. However, the theory did not hold up. Reports dating back to the 1940s showed that giving breast cancer patients estrogen also seemed to stop growth of the disease. In fact, before the emergence of tamoxifen, estrogen was routinely administered to postmenopausal women—or those no longer producing estrogen on their own—to combat the disease. Approximately 30% of patients responded favorably to the treatment.

More strikingly, when Doug Wolf, a graduate student in Jordan's laboratory in the 1990s, transplanted tamoxifen-resistant tumors from mouse to mouse and treated the animals with estrogen, he found that the tumors shrank (23, 24). "Estrogen didn't stimulate the growth of these tumors anymore. It killed them. They just melted away," Jordan recalls. "But we still didn't know the underlying mechanism of how that happened."

So Jordan set out to find out how the same hormone responsible for activating breast cancer also kills it off. He ultimately hopes to develop a new treatment approach for breast cancer patients who have grown resistant to tamoxifen. In his Inaugural Article, Jordan evaluates genetic changes to estrogen-starved breast cancer cells during the first week of estrogen therapy. The changes were striking, he says. The endoplasmic reticulum, or internal structure of the cell, quickly grew inflamed, triggering the cell's death. Moreover, Jordan found that cancer cell death occurred with relatively low doses of estrogen (6).

"In bodies that have been starved of estrogen, the hormone comes back as a jet fuel," Jordan says. That fuel overwhelms the estrogen receptor in breast cancer cells, causing them to invoke the "death signal." Jordan's finding suggests that it might make sense to treat women with tamoxifen-resistant tumors or those several years beyond menopause with low doses of estrogen. In estrogen-starved women, "The dramatic cell kill I get with estrogen is better than anything I saw with tamoxifen," Jordan says.

Living Legend

Growing up, Jordan says he did not have typical kid hobbies. Besides tinkering in his bedroom laboratory, he says, he loved ancient history. "I went on archaeological digs when I was a teenager in England," Jordan recalls.

His fondness for historical precedent, he says, is critical to his success as a pharmacologist. For the better part of a century, he says, nobody could understand why estrogen killed breast cancer in a certain subset of women. However, Jordan remembered Haddow's research from the 1940s and his graduate

student's serendipitous finding with tamoxifen-resistant tumors from the 1990s. "I believe that we're all part of this continuum. We're in a relay race and we've got to know where we've come from to show us where we're going," he says.

That focus has earned Jordan innumerable awards, but the honor for which Jordan remains most proud is one bestowed upon him by Northwestern University and the family of the late Diana, Princess of Wales. A longtime supporter of women's health initiatives, Princess Diana came to Chicago to support a symposium hosted by *People* magazine on

women's health and breast cancer. Jordan organized the event, and the two became friends. When Princess Diana died in a car accident in 1997, her family suggested establishing a professorship in her honor, earning Jordan the title Diana Princess of Wales Professor of Cancer Research at Northwestern University.

Chance encounters and obsession, Jordan says only half in jest, are key to his success. "Early on, I developed key concepts, and like a dog with a bone I never let those concepts go."

Sujata Gupta, *Freelance Science Writer*

- Jordan VC (1976) Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer* 12:419–424.
- Kiang DT, Kennedy BJ (1977) Tamoxifen (antiestrogen) therapy in advanced breast cancer. *Ann Intern Med* 87: 687–690.
- Jordan VC (2008) Tamoxifen: Catalyst for the change to targeted therapy. *Eur J Cancer* 44:30–38.
- Berry DA, et al. (2006) Modeling the impact of treatment and screening on U.S. breast cancer mortality: A Bayesian approach. *J Natl Cancer Inst Monogr* 36: 30–36.
- Haddow A (1970) David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26:737–754.
- Ariazi EA, et al. (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci USA* 108:18879–18886.
- Harper MJK, Walpole AL (1967) A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *J Reprod Fertil* 13:101–119.
- Klopper A, Hall M (1971) New synthetic agent for the induction of ovulation: Preliminary trials in women. *BMJ* 1:152–154.
- Jordan VC, Dix CJ, Allen KE (1979) The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. *Adjuvant Therapy of Cancer II*, eds Salmon SE, Jones SE (Grune & Stratton, Philadelphia), pp 19–26.
- Jordan VC, Allen KE (1980) Evaluation of the anti-tumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 16:239–251.
- Davies C, et al.; Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials. *Lancet* 378:771–784.
- Jordan VC, Phelps E, Lindgren JU (1987) Effects of antiestrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10:31–35.
- Love RR, et al. (1990) Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst* 82:1327–1332.
- Love RR, et al. (1992) Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 326:852–856.
- Ettinger B, et al.; Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators (1999) Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: Results from a 3-year randomized clinical trial. *JAMA* 282:637–645.
- Jordan VC (2006) Optimising endocrine approaches for the chemoprevention of breast cancer beyond the Study of Tamoxifen and Raloxifene (STAR) trial. *Eur J Cancer* 42:2909–2913.
- Cummings SR, et al. (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281:2189–2197.
- Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC (1988) Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48:812–815.
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 365:1687–1717.
- Vogel VG, et al. (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295: 2727–2741.
- Vogel VG, et al. (2010) Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 3:696–706.
- Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183–5187.
- Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results in Cancer Research* (Springer-Verlag, Heidelberg) Vol 127, pp 23–33.
- Yao K, et al. (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6: 2028–2036.

Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time

Eric A. Ariazi^a, Heather E. Cunliffe^b, Joan S. Lewis-Wambi^a, Michael J. Slifker^a, Amanda L. Willis^{b,1}, Pilar Ramos^b, Coya Tapia^{b,2}, Helen R. Kim^{a,c,3}, Smitha Yerrum^{a,4}, Catherine G. N. Sharma^a, Emmanuelle Nicolas^a, Yoganand Balagurunathan^b, Eric A. Ross^a, and V. Craig Jordan^{a,c,3,5}

^aFox Chase Cancer Center, Philadelphia, PA 19111; ^bTranslational Genomics Research Institute, Phoenix, AZ 85004; and ^cLombardi Comprehensive Cancer Center, Washington, DC 20057

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2009.

Contributed by V. Craig Jordan, September 14, 2011 (sent for review June 21, 2011)

In laboratory studies, acquired resistance to long-term antihormonal therapy in breast cancer evolves through two phases over 5 y. Phase I develops within 1 y, and tumor growth occurs with either 17 β -estradiol (E₂) or tamoxifen. Phase II resistance develops after 5 y of therapy, and tamoxifen still stimulates growth; however, E₂ paradoxically induces apoptosis. This finding is the basis for the clinical use of estrogen to treat advanced antihormone-resistant breast cancer. We interrogated E₂-induced apoptosis by analysis of gene expression across time (2–96 h) in MCF-7 cell variants that were estrogen-dependent (WS8) or resistant to estrogen deprivation and refractory (2A) or sensitive (5C) to E₂-induced apoptosis. We developed a method termed differential area under the curve analysis that identified genes uniquely regulated by E₂ in 5C cells compared with both WS8 and 2A cells and hence, were associated with E₂-induced apoptosis. Estrogen signaling, endoplasmic reticulum stress (ERS), and inflammatory response genes were overrepresented among the 5C-specific genes. The identified ERS genes indicated that E₂ inhibited protein folding, translation, and fatty acid synthesis. Meanwhile, the ERS-associated apoptotic genes Bcl-2 interacting mediator of cell death (BIM; BCL2L11) and caspase-4 (CASP4), among others, were induced. Evaluation of a caspase peptide inhibitor panel showed that the CASP4 inhibitor z-LEVD-fmk was the most active at blocking E₂-induced apoptosis. Furthermore, z-LEVD-fmk completely prevented poly (ADP-ribose) polymerase (PARP) cleavage, E₂-inhibited growth, and apoptotic morphology. The up-regulated proinflammatory genes included IL, IFN, and arachidonic acid-related genes. Functional testing showed that arachidonic acid and E₂ interacted to superadditively induce apoptosis. Therefore, these data indicate that E₂ induced apoptosis through ERS and inflammatory responses in advanced antihormone-resistant breast cancer.

aromatase inhibitor | antihormonal resistance | estrogen receptor | gene expression microarrays | selective estrogen receptor modulator

Elucidation of the basic structure function relationships of synthetic estrogens based on either stilbene (1) or triphenylethylene (2) was a landmark achievement that continues to have major therapeutic implications to this day. The first successful chemical therapy for the treatment of any cancer was the use of high-dose synthetic estrogen for the treatment of metastatic breast cancer (3). Response rates for patients who were more than a decade beyond menopause were about 30%. Importantly, treatment near menopause was ineffective, and therefore, tumor responsiveness was related to the duration of estrogen deprivation. In 1970, Alexander Haddow commented that “the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases [with oestrogen] has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms con-

tinues to elude us” (4). High-dose estrogen therapy using diethylstilbestrol (DES) remained the standard of care for the treatment of metastatic breast cancer in postmenopausal women for 30 y (1950s to late 1970s in the United States). However, triphenylethylene-based estrogens evolved into nonsteroidal antiestrogens (5), where the initial interest focused on their potential as postcoital antifertility agents. This application failed, and the compounds were subsequently reinvented as antiestrogens targeted to estrogen receptor (ER) for the treatment of all stages of breast cancer (6, 7). Subsequently, the nonsteroidal antiestrogens would again evolve and be reinvented as selective ER modulators (SERMs) (8). This new drug class exploited the observations that they blocked breast cancer development and growth as antiestrogens but lowered circulating cholesterol and maintained bone density as estrogens. This finding led to the idea that the treatment and prevention of osteoporosis would simultaneously prevent breast cancer (5, 9). Raloxifene is the first SERM of the class used to prevent both osteoporosis and breast cancer (10, 11).

The strategy of targeting ER and using long-term adjuvant tamoxifen therapy for breast cancer treatment (7) has increased 15-y survival rates (12, 13) and contributed significantly to the national reduction breast cancer mortality (14). From 1975 to 1990, breast cancer mortality rates held roughly steady, but from 1990 to 2000, they declined by 19.6%. It is estimated that about two-thirds of this reduction is because of therapy and one-third is because of mammography screening. Specifically, in ER-positive tumors, 5 y of tamoxifen therapy was estimated to have reduced the hazard of breast cancer mortality by 37% (14). Tamoxifen remains the antihormone treatment of choice for the adjuvant treatment of breast cancer in premenopausal patients, despite the development

Author contributions: E.A.A., H.E.C., J.S.L.-W., and V.C.J. designed research; E.A.A., H.E.C., J.S.L.-W., A.L.W., P.R., C.T., H.R.K., S.Y., C.G.N.S., and E.N. performed research; E.A.A., M.J.S., Y.B., and E.A.R. contributed new reagents/analytic tools; E.A.A., H.E.C., M.J.S., and Y.B. analyzed data; and E.A.A., H.E.C., M.J.S., and V.C.J. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE29917).

¹Present address: Division of Molecular Medicine and Genetics, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109.

²Present address: Institute for Pathology, University Hospital Basel, 4103 Basel, Switzerland.

³Present address: Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC 20057.

⁴Present address: Laboratory of Molecular Oncology, Van Andel Research Institute, Grand Rapids, MI 49503.

⁵To whom correspondence should be addressed. E-mail: vcj2@georgetown.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1115188108/-DCSupplemental.

of the aromatase inhibitors (AIs) for the adjuvant treatment of postmenopausal patients with ER-positive breast cancer. The AIs provide a modest, but significant, improvement in disease-free survival for patients and a significant decrease in the incidence of both endometrial cancer and thromboembolism associated with tamoxifen therapy in postmenopausal women (15). Nevertheless, tamoxifen remains an important and cheap lifesaving drug, available in countries without a sophisticated healthcare infrastructure.

Despite the ability of long-term adjuvant antihormone therapy to enhance breast cancer patient survivorship, the consequence of any sustained therapy to control tumor growth is the development of resistance. Studies in vivo with MCF-7 cells inoculated into athymic mice showed that, although tamoxifen initially blocked tumor growth, eventually tumors would grow, despite continued tamoxifen treatment (16). Similar studies showed that tamoxifen, in fact, stimulated growth of resistant MCF-7 tumors (17). A new form of acquired drug resistance was described for breast cancer that grew in response to ER activation through either tamoxifen or the natural ligand 17β -estradiol (E_2). This finding explained the observed resistance to tamoxifen in ER-positive metastatic breast cancer patients after ~ 1 –2 y of therapy but was inconsistent with the clinical observation that patients with stages I and II breast cancer could be routinely treated with 5 or more y of adjuvant tamoxifen therapy without developing tumor recurrence. A possible explanation would emerge from studies of acquired resistance to antihormone therapy that, at the same time, would expose a vulnerability of breast cancer cells and explain the mechanism of high-dose estrogen therapy for the treatment of breast cancer.

The continuous passage of MCF-7 tumors for more than 5 y in tamoxifen-treated athymic mice results in a reconfiguration of survival signaling pathways. Although tumors remain tamoxifen stimulated for growth, physiologic E_2 now causes rapid tumor regression rather than growth (18, 19). Indeed, some tumors that regress and then regrow during continuous E_2 treatment are exclusively E_2 -dependent, because tamoxifen or E_2 withdrawal will impair tumor growth (19). The evolution of acquired resistance to SERMs (20) naturally raised the concern of the development of resistance to the new standard of care for adjuvant treatment of ER-positive breast cancer in postmenopausal patients, the AIs.

Parallel studies to replicate the clinical expression of acquired resistance to estrogen deprivation (i.e., resistance to an AI) (21, 22) were initiated in vitro 20 y ago using ER-positive MCF-7 breast cancer cells. When cells were grown under long-term estrogen-deprived conditions (>1 y), cells lost their dependency on estrogen for proliferation but maintained expression of ER. Subsequent studies of E_2 action on the growth of long-term estrogen-deprived MCF-7 cells in vitro at high (23) and low concentrations in vitro and in vivo (24, 25) indicated that the concept of “an estrogen purge” (19) to destroy antihormone-resistant cells could be applied to the treatment of breast cancer. This concept has now been translated to clinical trials.

A pivotal study of high-dose DES therapy (15 mg daily) in 32 patients with metastatic breast cancer who had been treated exhaustively with antihormonal therapies produced a 30% objective response rate (26). There were 4 of 32 complete responses, and one patient maintained a complete response for an additional 7 y, even after stopping estrogen (27). A recent study in patients whose breast cancer had responded but then failed AI treatment (28) showed that low-dose E_2 treatment (6 mg daily) would produce the same clinical benefit as high-dose E_2 (30 mg daily) but with fewer toxic side effects. Thus, laboratory observations with low doses of estrogen treatment translate to clinical practice, and a mechanism is now emerging to explain the original observations by Haddow (3, 4). The goal of future translational research is to discover molecular mechanisms to amplify the estrogen-induced apoptotic trigger.

The question arises as to the precise sequence of events that lead to E_2 -induced apoptosis. By describing and defining these molecular events, refractory cells may be manipulated to respond to estrogen-induced apoptosis. To begin to address the question, we have developed a series of MCF-7 variants that are either estrogen-dependent for growth (MCF-7:WS8 cells) (29–31) or resistant to estrogen deprivation (ED) and refractory (MCF-7:2A) (25, 30, 31) or sensitive (MCF-7:5C) (24, 29, 32) to E_2 -induced apoptosis. We previously reported changes in gene expression among these cell lines by Affymetrix-based microarray analysis under estrogen-free conditions (33). Thus, these identified differentially expressed genes were associated with progression to an ED-resistant phenotype. We have also recently reported a proteomic analysis of 5C compared with WS8 cells after 2 h of E_2 exposure to identify proteins that may initiate apoptosis (34). Here, we seek to identify genes differentially regulated by E_2 over a 2–96 h time course, which overlaps with actively occurring apoptosis. Therefore, we interrogated these models for changes in E_2 -regulated global gene expression as a function of time using Agilent 4×44 K oligonucleotide microarrays. We developed a method termed differential area under the curve (dAUC) analysis to identify genes that exhibited significantly altered regulation by E_2 across time specifically in the apoptosis-sensitive 5C cells compared with both the estrogen-dependent WS8 and apoptosis-refractory 2A cells. Examination of the identified 5C-specific genes and functional testing indicated that E_2 -elicited endoplasmic reticulum stress (ERS) and inflammatory stress responses that led to apoptosis.

Results and Discussion

Cell Line Characterization. Before gene expression microarray studies were carried out, the estrogen-dependent WS8 (29–31), ED-resistant but apoptosis-refractory 2A (25, 30, 31), and apoptosis-sensitive 5C cells (24, 29, 32) were characterized to confirm previously reported growth responses, biomarker status, and estrogen response element (ERE)-regulated transcriptional activity (*SI Results and Discussion*, *SI Methods*, and *Fig. S1*). The apoptotic responses of 5C cells to E_2 were also characterized according to loss of plasma membrane integrity (*SI Results and Discussion*, *SI Methods*, and *Fig. S2*). The 5C cells exhibited an EC_{50} for apoptosis of 3×10^{-11} M E_2 after 96 h of exposure (*Fig. S2B*). Additionally, 10^{-9} M E_2 , the concentration used for the microarray studies, caused apoptosis ranging from $\sim 30\%$ to 42% of the 5C cells, depending on the experiment (*Fig. S2 B and C*). The pure antiestrogen fulvestrant completely blocked apoptosis induced by E_2 and DES, showing that apoptosis was ER-dependent (*Fig. S2C*).

Global Gene Expression Across Time. To identify genes and pathways/processes associated with E_2 -induced apoptosis, differential regulation of global gene expression in response to E_2 was interrogated in ED-resistant/apoptotic-sensitive 5C cells vs. estrogen-dependent WS8 and ED-resistant/apoptotic-refractory 2A cells. Each cell line was treated with 10^{-9} M E_2 or vehicle control over a 96-h time course consisting of seven time points (2, 6, 12, 24, 48, 72, and 96 h) using six biological replicates per condition. cRNA probes from individual E_2 -treated samples were competitively hybridized against time-matched pooled control probes using two-color Agilent 4×44 K human oligonucleotide microarrays. The resulting gene expression values were \log_2 ratios of mRNA levels in E_2 /control-treated cells that, when plotted across time, form a curve. A measure of change in E_2 -mediated regulation of expression over a defined time interval was then calculated as the difference in AUCs or dAUCs for a given gene between two cell lines. Genes that showed a 50% change in AUCs between two cell lines (corresponding to a dAUC = 0.58 on a \log_2 scale) at a P value < 0.00005 (*Methods* has details on P value determination) were defined as significantly different. The dAUC method was

applied to identify differentially regulated genes at 2–96, 2–24, and 24–96 h to identify overall, relatively early, and late-responding genes, respectively.

To identify genes specifically associated with E₂-induced apoptosis, genes were selected with regulation that differed significantly with E₂ in the 5C cells vs. both the WS8 and 2A cells. A total of 1,142 genes were identified as significantly differentially regulated by E₂ specifically in the 5C cells (Dataset S1). These genes were examined for overrepresentation of those genes mapping to a particular curated pathway/network (Fig. S3A). As expected, estrogen signaling and apoptosis genes were significantly enriched. Within the apoptosis category, ERS was the most enriched apoptosis subcategory (Fig. S3B). Inflammatory response genes were also enriched. The overlapping distribution of genes mapping to estrogen signaling (Dataset S2), apoptosis (Dataset S3), and inflammatory responses (Dataset S4) is shown in the Venn diagram in Fig. S3C.

Estrogen Signaling Genes. Estrogen signaling genes selectively regulated by E₂ in 5C cells relative to both WS8 and 2A cells are listed in Dataset S2, and examples discussed are shown in Fig. 1. Multiple genes were differentially regulated by E₂ in 5C cells compared with WS8 and 2A cells, which would diminish ERα activity and hence, the apoptotic stimulus. For example, genes that negatively modulate ERα activity (i.e., AR, CYP1B1, FHL2, HSD17B11, INHBA, NR2F1/COUP-TF1, SNAI1/Snai1, and THRA/TRα) were selectively up-regulated, whereas those genes that promote ERα activity were selectively down-regulated (AREG, CAV1, and PIK3CB) by E₂ in 5C cells. The up-regulated estrogen metabolizing enzymes CYP1B1 and HSD17B11 would decrease intracellular E₂ pools. SETD7/SET7/SET9 methylates ER to stabilize the protein (Dataset S5, ref. 1); hence, its down-regulation by E₂ in 5Cs would accelerate ERα protein degradation. ERα activity would be suppressed by up-regulation of transcription factors that repress ERα RNA expression (i.e., FHL2 and Snai-1) (Dataset S5, refs. 2 and 3) or compete with ERα for binding-extended ERE half-sites, which overlap with many natural EREs (i.e., COUP-TF1 and TRα) (Dataset S5, refs. 4 and 5). AR failed to down-regulate in response to E₂ in 5C cells, allowing greater AR activity. AR and ERα interact in complexes, and androgens inhibit E₂-stimulated growth of MCF-7 cells (Dataset S5, ref. 6); thus, AR can oppose ERα. ERα

activity can also be suppressed by activin-A, a TGFβ superfamily ligand, in a SMAD3-dependent manner in MCF-7 cells (Dataset S5, ref. 7). Both INHBA and SMAD3 were selectively induced in 5C cells, and INHBA homodimerizes to form activin-A, which signals to SMAD3; SMAD3 interacts with ERα at promoters to repress transcription. AREG and PIK3CB failed to increase in response to E₂ in 5C cells. This failure to increase may have prevented increased ERα activity, because AREG activates EGFR, which leads to ERα-Ser118 phosphorylation, and PIK3CB is the catalytic subunit of PI3K, which through Akt, targets ERα-Ser167 phosphorylation (Dataset S5, ref. 8). CAV1 expression also failed to increase, which again prevents increased ERα activity, because CAV1 interacts with and promotes activity of membrane-localized ERα (Dataset S5, ref. 9). However, not all of ERα's activities were suppressed. In particular, ERα interacts with and directs transcription through AP-1 transcription complexes in addition to EREs (Dataset S5, ref. 10). AP-1 complexes consist of FOS, JUN, and JUND subunits, which were all selectively induced by E₂ in 5C cells [FOS and JUN were verified by quantitative PCR (qPCR) in Fig. S4]. Importantly, AP-1 complexes play important roles in apoptosis and inflammatory responses (discussed later), and thus, ERα interaction with AP-1 provides a mechanism for E₂ to target such genes.

Apoptosis Genes. The identified apoptosis genes are listed in Dataset S3, and discussed examples are shown in Fig. 2. Enrichment analysis indicated ERS-mediated apoptosis as the top-scoring individual pathways within the apoptosis category (Fig. S3). The endoplasmic reticulum is a key site for protein folding. When cellular stresses perturb energy levels, the redox state, or Ca²⁺ concentrations, unfolded proteins accumulate and protein aggregation occurs; this condition is referred to as ERS (Dataset S5, refs. 11 and 12). To relieve ERS, an unfolded protein response (UPR) is triggered by the chaperone HSPA5/GRP78/BiP. In addition to binding unfolded proteins, GRP78 binds and prevents oligomerization of the endoplasmic reticulum transmembrane receptors EIF2AK3/PERK, IRE1α/ERN1, and ATF6. When unfolded proteins accumulate, GRP78 is released from binding the transmembrane receptors, allowing them to oligomerize and autophosphorylate to initiate a UPR signal. The UPR signals to attenuate protein translation, induce expression of additional chaperones, and export misfolded proteins to the cytosol for degradation. If the UPR fails

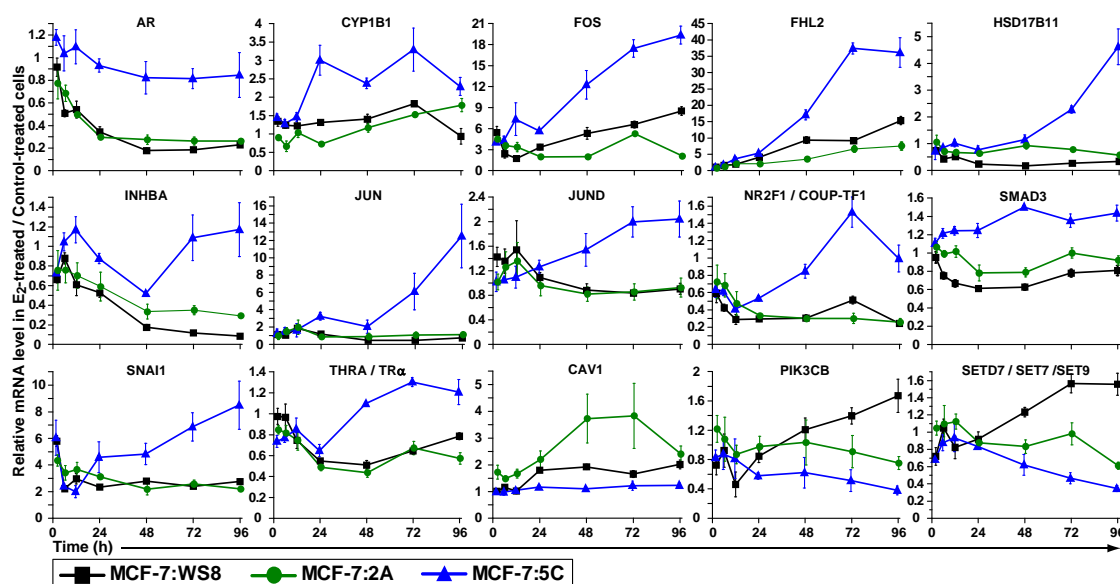


Fig. 1. Examples of estrogen signaling genes. Full annotation, dAUC values, and *P* values of all estrogen signaling genes are given in Dataset S2.

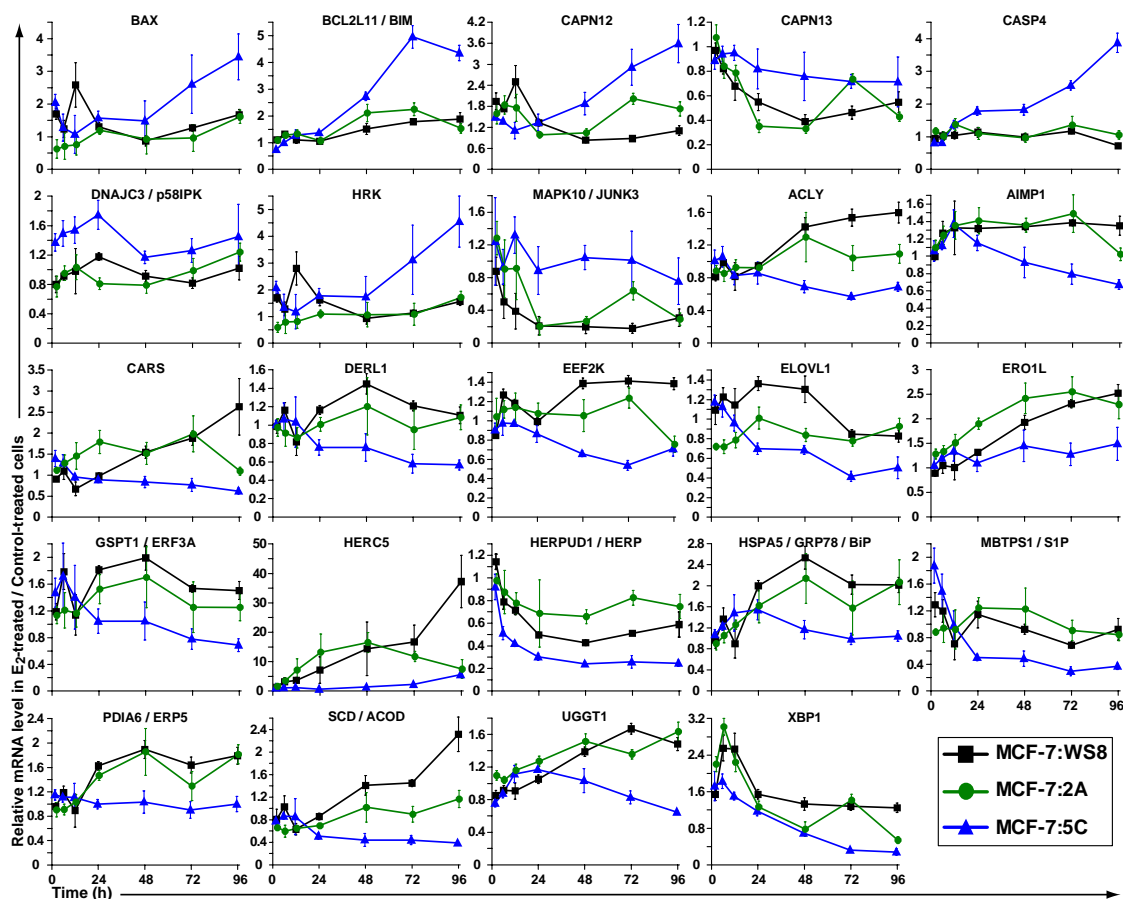


Fig. 2. Examples of apoptosis genes. Full annotation, dAUC values, and *P* values of all apoptosis genes are given in Dataset S3.

to relieve the stress, the function of the UPR switches from promoting cell survival to promoting cell death. Thus, excessive or prolonged ERS typically induces apoptosis.

Growth stimulation of hormonally responsive cells by E_2 leads to increases in requirements for folding nascent polypeptides and clearance of malformed proteins. However, in 5C cells compared with WS8 and 2A cells, E_2 -regulated expression changes indicated a deficiency in these functions. In 5C cells, E_2 failed to sufficiently up-regulate endoplasmic reticulum-localized protein folding genes, including GRP78 (verified by qPCR in Fig. S4), ERO1L, PDIA6, and UGGT1. Cytoplasmic protein folding genes, including HSP90AB1/HSP90B, PPIAL4A, and PPIF (also FKBP10), also failed to up-regulate. Additionally, in 5C cells, E_2 preferentially down-regulated HERPUD1/HERP1 and DERL1, factors that promote degradation of endoplasmic reticulum-resident proteins. A deficiency in up-regulating UPR genes in 5C cells may have resulted in part by the pronounced E_2 -mediated repression of MBTPS1/S1P, which cleaves ATF6, activating its translocation to the nucleus to induce transcription of UPR genes, including XBP1. Thus, decreased S1P may have led to decreased ATF6 and XBP1 activity, thereby preventing induction of multiple UPR genes.

E_2 -mediated gene expression alterations in 5C cells indicated widespread inhibition of protein translation compared with E_2 -treated WS8 and 2A cells. Within 2 h, E_2 had up-regulated DNAJC3/p58^{IPK}, which binds to and inactivates EIF2AK3/PERK, leading to reduced global translational initiation (Dataset S5, ref. 12). The aminoacyl tRNA synthetase interacting protein AIMP1 and tRNA synthetases, including CARS (also LARS, SARS, and YARS) failed to increase in response to E_2 in 5Cs. Other trans-

lational factors that failed to induce in 5C cells include EEF2K and GSPT1/ERF3A (also EEF1A1, ETF1, and PABPC4).

Under severe ERS, the UPR can shut down lipogenesis as cells commit to death (Dataset S5, ref. 12). This was likely the case in E_2 -treated 5C cells since they showed a lack of induction of critical genes involved in fatty acid synthesis, including ACLY, SCD/ACOD, and ELOVL1. ACLY is the primary enzyme responsible for synthesis of acetyl-CoA, the basic building block of fatty acids. SCD introduces a C-C double bond in fatty acyl-CoA substrates, including stearoyl-CoA and palmitoyl-CoA, a key step in producing monounsaturated fatty acids. ELOVL1 condenses both saturated and monounsaturated fatty acids. Notably, SCD and ELOVL1 are localized to the endoplasmic reticulum membrane.

In response to severe ERS, specific BCL2 and Bcl-2 homology domain 3 (BH3) -only family members are targeted to initiate apoptosis (Dataset S5, ref. 11). Prototypical BCL2 inhibits cell death by binding and inactivating proapoptotic members such as BAX. BH3 only-containing proteins like BCL2L11/BIM indirectly activate BAX by binding BCL2 (through the BH3 motif), thereby releasing BAX from the complex. BAX then permeabilizes the mitochondrial outer membrane, allowing cytochrome C release to the cytoplasm. Under ERS, BAX also interacts with and activates IRE1 α . IRE1 α then signals to JNK to simultaneously activate BIM and inhibit BCL2 (Dataset S5, ref. 11). A variety of ERS inducers stimulate BIM expression, and BIM is essential in ERS-induced apoptosis in a wide range of cell types (Dataset S5, ref. 13). This apoptotic pathway was likely activated by E_2 in 5C cells. E_2 failed to repress MAPK10 (JNK3) in 5C cells, indicating higher JNK3 activity. Meanwhile, E_2 selectively up-regulated BAX, BIM (verified

by qPCR in Fig. S4), and another BH3-only proapoptotic factor, HRK (also BBC3/PUMA but PUMA did not make the significance cutoff) (Fig. S5). Importantly, E₂ repressed BCL2 in 5C cells but induced it in WS8 cells. However, E₂ also repressed BCL2 in 2A cells, and therefore, it was not a 5C-specific gene (Fig. S5). We previously verified the importance of BAX and BIM by showing that they were selectively induced by E₂ at the protein level in 5C vs. WS8 cells and that their depletion by RNAi blocked E₂-induced apoptosis (31). Therefore, ERS may have triggered mitochondrial-mediated apoptotic cell death in E₂-treated 5C cells.

After prolonged ERS, specific caspases are activated to enact cell death. Examination of the caspases revealed that only CASP4 met the stringent statistical significance criteria in the microarray data. CASP1, CASP5, and CASP8 also showed up-regulation in 5C cells but did not meet our significance threshold (Fig. S5). CASP4 along with CASP1 and CASP5 are inflammatory caspases, because they are involved in cytokine maturation (Dataset S5, ref. 14). CASP4 specifically localizes to the endoplasmic reticulum and undergoes cleavage in response to ERS-inducing agents/proteins but not other apoptotic agents, and its blockade using z-LEVD-fmk or depletion by RNAi can prevent endoplasmic stress-induced apoptosis in multiple model systems (Dataset S5, refs. 15–20). Importantly, CASP4 autoactivates by dimerizing and undergoing interdomain cleavage (Dataset S5, ref. 21), and thus, simply overexpressing CASP4 is sufficient to induce cleavage of downstream caspases (Dataset S5, ref. 22) and cause apoptosis (Dataset S5, ref. 23). Under ERS, CASP4 can also be activated by calpain (Dataset S5, refs. 24 and 25), and CAPN12 and CAPN13 were selectively up-regulated in 5C cells.

Inflammatory Response Genes. The inflammatory response genes are listed in Dataset S4, and discussed examples are shown in Fig. 3. In 5C cells, E₂ elicited up-regulation of many proinflammatory cytokine/cytokine receptors, including IL-4R (verified by qPCR in Fig. S4), IL-6R, IL-6ST/gp130, IL-17RD/Sef, and VEGFA. IL-4R was induced with early kinetics, indicating that it may be a primary response. IL-6R was up-regulated shortly after IL-4R, whereas IL-6ST/gp130, also an IL-4R subunit, was already up-regulated by 2 h. Hence, IL-6 signaling was likely activated in 5Cs. IL-17RD/Sef not only mediates IL-17 signaling, but its overexpression also leads to JNK activation and apoptosis (Dataset S5, ref. 26), which links inflammatory responses and ERS. VEGFA also leads to activation of JNK in tamoxifen-resistant MCF-7 cells (Dataset S5, ref. 27). An IFN response was likely activated, because the IFN IFNL1 and the IFN-responsive genes IFI6 and IFI16 (Dataset S3) were up-regulated. CASP4 can also be induced by IFN (Dataset S5, ref. 28).

A number of other proinflammatory genes, such as CEBPB, NTN1 (verified by qPCR in Fig. S4), and UNC5C, were selectively up-regulated in E₂-treated 5C cells with relatively early kinetics, indicating possible mechanistic roles. CEBPB is important in induction of IL-6, is activated by ERS (Dataset S5, ref. 29), is required for nuclear import of the key ERS protein CHOP/GADD153 (Dataset S5, ref. 30), and enhances NF-κB signaling (Dataset S5, refs. 31 and 32). NTN1 is a secreted inflammatory marker, but it protects tissues from inflammatory injury by suppressing cytokine production, repulsing leukocyte infiltration, and acting as an antiinflammatory and antiapoptotic ligand of its receptors DCC and the UNC-5 family members (Dataset S5, refs. 33 and 34). In the context of E₂-induced apoptosis, NTN1 may have been up-regulated to limit or resolve the inflammatory response. Interestingly, E₂ rapidly down-regulated UNC5C in WS8 and 2A cells within 6 h but failed to do so in 5C cells, resulting in higher UNC5C expression. UNC5C may have a proinflammatory role, because synovial cells from patients with rheumatoid arthritis and osteoarthritis dramatically overexpress UNC5C (769-fold) compared with those cells of healthy donors (Dataset S5, ref. 35).

Arachidonic acid (AA; 20:4n-6) is a polyunsaturated fatty acid that plays a key role as an inflammatory mediator. Enzymes involved in AA biosynthesis were up-regulated by E₂ in 5C cells, including FADS1 (verified by qPCR in Fig. S4), FADS3, PLA2G10, PLCD3, MGLL/MAGL, PPAP2A/LPP1 (verified by qPCR in Fig. S4), and SGMS1/SMS1. FADS3 and FADS1 catalyze the first and last steps in AA biosynthesis by introducing C-C double bonds in linoleic acid, producing γ-linolenic acid (18:3n-6), and dihomo-γ-linolenic acid (20:3n-6), producing AA. PLA2s hydrolyze phospholipids, releasing AA, whereas PLCD3 cleaves AA from diacylglycerol. MGLL converts monoacylglycerides such as 2-arachidonoylglycerol to free fatty acids including AA. PPAP2A/LPP1 converts phosphatidic acid to diacylglycerol, providing increased substrate levels for PLCD3 to release AA. As an inflammatory mediator, AA is used as a precursor by cyclooxygenase and lipoxygenase to generate inflammatory prostaglandins and leukotrienes, respectively. However, the cyclooxygenase pathway was unlikely to have been involved in E₂-induced apoptosis, because induction of PTGES failed in 5C cells compared with WS8 and 2A cells. In hormone-dependent breast cancer cells, E₂ is known to induce PTGES expression through an ERE, which may promote breast cancer proliferation, because the increased prostaglandin E₂ may enhance aromatase expression and also promote local productions of estrogens (Dataset S5, ref. 36). Thus, a failure to induce PTGES may, ultimately, have served to prevent any potential increases in estrogen concentrations in 5C cells. Considering that ERS likely led to a block of fatty acid synthesis and conversion to monounsaturated fatty acids (i.e., no induction of ACLY and SCD), the selective increases in AA-related genes likely indicate the importance of AA in promoting an inflammatory response in E₂-induced apoptosis.

Cross-Talk Between ERS and Inflammatory Stress. As mentioned previously, ERS and inflammatory pathways intersect. The key ERS genes IRE1α, ATF6, and PERK can all activate NF-κB, which serves as a master regulator of inflammatory response gene transcription (Dataset S5, refs. 12 and 37). Many of the identified cytokine/cytokine receptors signal through NF-κB pathways. Other genes selectively induced by E₂ in 5C cells, including BCL10 (Dataset S5, ref. 38), CXXC5 (Dataset S5, ref. 39), LTB (verified by qPCR in Fig. S4 and Dataset S5, ref. 40), and ITGB2 (Dataset S4; Dataset S5, ref. 41), activate NF-κB signaling as well. Additionally, SETD7/SET7/SET9, which negatively regulates NF-κB activity by methylating the RelA subunit to induce its degradation (Dataset S5, ref. 42), was down-regulated by E₂ in 5Cs (Fig. 1). Furthermore, multiple 5C-specific genes are NF-κB-responsive, including BIM (Dataset S5, refs. 43 and 44), CASP4 (Dataset S5, ref. 45), CEBPB (Dataset S5, ref. 46), CP (Dataset S4; Dataset S5, ref. 47), NTN1 (Dataset S5, ref. 48), and VEGFA (Dataset S5, ref. 49). Moreover, ERα and NF-κB can interact to transcriptionally regulate promoters, providing a direct mechanism for E₂ to target a diverse array of inflammatory and apoptotic genes. Therefore, NF-κB signaling was very likely involved in E₂-induced apoptosis, and we are pursuing this hypothesis in future studies.

ERS also intersects with inflammatory responses through JNK. As mentioned, the ERS sensor IRE1α (Dataset S5, ref. 12) and the IL receptor 17RD/Sef can activate JNK (Dataset S5, ref. 26). The orphan TNF receptor TNFRSF19/TAJ, which failed to down-regulate in response to E₂ in 5C cells, also activates JNK (Dataset S5, ref. 50). JNK then phosphorylates AP-1 complexes to induce expression of inflammatory response genes (Dataset S5, ref. 12). As mentioned earlier, the AP-1 subunits JUN, JUND, and FOS were selectively induced in E₂-treated 5C cells.

Functional Involvement of AA and CASP4 in E₂-Induced Apoptosis.

The involvement of ERS and inflammatory stress in E₂-induced apoptosis was functionally examined. We first tested

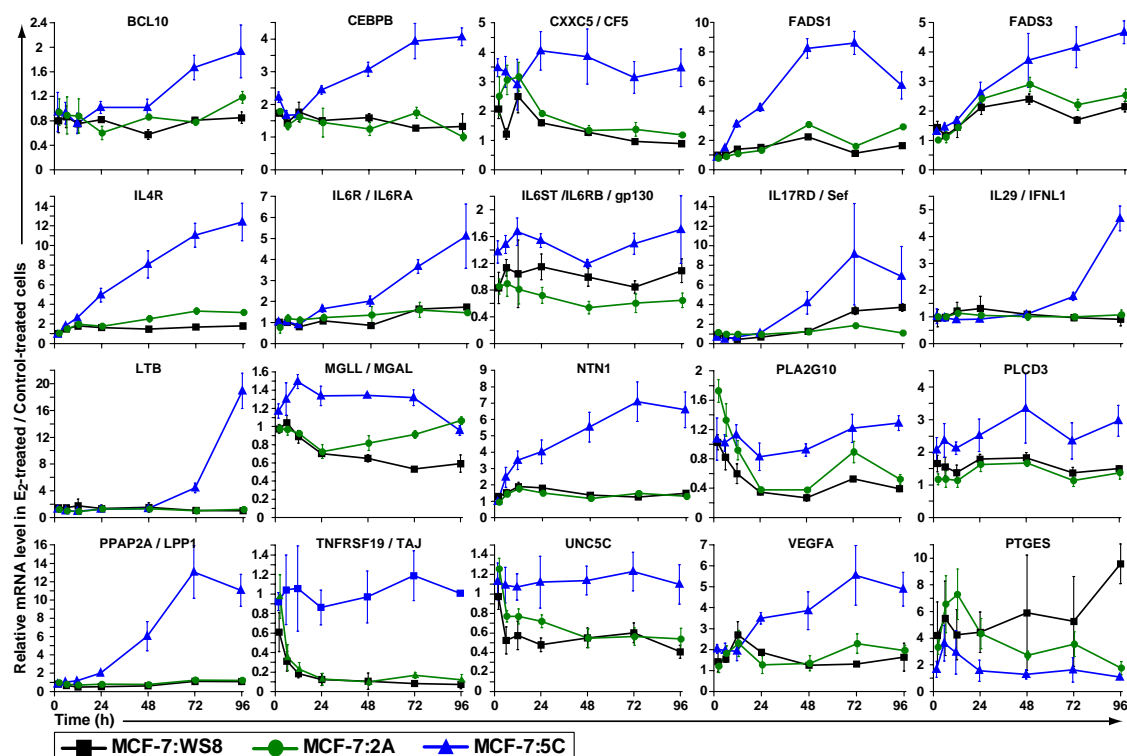


Fig. 3. Examples of inflammatory response genes. Full annotation, dAUC values, and *P* values of all inflammatory response genes are given in [Dataset S4](#).

whether E_2 -induced apoptosis could be promoted by AA. AA was chosen, because (i) it is widely recognized as a proinflammatory agent; (ii) it induces apoptosis ([Dataset S5](#), ref. 51), at least in part by depleting the endoplasmic reticulum of Ca^{2+} and inhibiting protein translation, thereby likely eliciting ERS ([Dataset S5](#), ref. 52); (iii) it can activate NF- κ B in mammary epithelial cells ([Dataset S5](#), ref. 53); and (iv) several genes, which increase AA levels (e.g., FADS1 and PLA2G10), were up-regulated in response to E_2 in 5C vs. WS8 and 2A cells. 5C cells were exposed to varying concentrations of both AA and E_2 in a factorial design, and then, apoptosis was measured by flow cytometric analysis of YO-PRO-1 and 7-aminoactinomycin D staining (Fig. 44). Because E_2 -induced apoptosis occurs maximally with 10^{-9} M E_2 after 96 h of exposure, E_2 was used at low concentrations of 2.5 and 5×10^{-11} M, and apoptosis was assayed at 72 h to allow observation of potential additional AA effects. The combination of AA plus E_2 at all varied concentrations increased the percentage of apoptotic plus dead cells in a greater than additive manner relative to either agent alone. Fitting the data to a multiple regression model showed the rate of increase (slope) in apoptotic plus dead cells progressively and significantly increased comparing E_2 alone with $E_2 + 10 \mu$ M AA or $E_2 + 20 \mu$ M AA. Therefore, AA and E_2 interacted to superadditively induce apoptosis, indicating that their pathways functionally intersect.

The importance of CASP4 was evaluated using a panel of irreversible caspase peptide inhibitors selectively targeting caspases-1 to -9 (except CASP3, which is not expressed in MCF-7 cells) ([Dataset S5](#), ref. 54). 5C cells were treated with 10^{-9} M E_2 plus each caspase inhibitor as indicated for 96 h to induce apoptosis, which was measured by altered plasma membrane permeability (Fig. 4B). The broad spectrum caspase inhibitor z-VAD-fmk was used as a positive control, because we previously reported that this inhibitor completely blocks E_2 -induced apoptosis (31), whereas the inactive inhibitor z-FA-fmk was used as a negative control. In an effort to prevent off-target caspase inhibition, the blockers were used at 10 μ M, which was the con-

centration that reduced apoptosis by approximately one-half by the pan inhibitor z-VAD-fmk. The most active inhibitor was the CASP4 blocker z-LEVD-fmk, which was slightly more effective than the pan CASP inhibitor (Fig. 4B). The CASP8 inhibitor z-IETD-fmk was the next most active blocker but was significantly less potent than z-LEVD-fmk (*P* value = 0.0026). Therefore, in an unbiased comparison of caspases-1 to -9, CASP4 was validated as functionally critical in E_2 -induced apoptosis.

The functional activity of CASP4 was also studied. Real-time qPCR and immunoblotting confirmed induction of CASP4 expression at the mRNA and protein levels, respectively, occurred specifically in 5C cells in response to E_2 (Fig. 5A and B). Importantly, in 5C cells, z-LEVD-fmk at 20 μ M completely blocked E_2 -induced PARP cleavage (Fig. 5B), reversed E_2 -inhibited growth (Fig. 5C), and prevented morphologic alterations associated with apoptosis in 5C cells (Fig. 5D). Because z-LEVD-fmk was used at 20 rather than 10 μ M, we do not discount the possibility that some caspases in addition to CASP4 were also inhibited and that other caspases could still play an important role. Yet, our data establishes a critical role for CASP4 in E_2 -induced apoptosis.

Concluding Remarks. We have interrogated E_2 -induced apoptosis by identifying differentially regulated genes across time associated with this process compared with E_2 -stimulated and -independent growth using a method we developed termed dAUC analysis. Overrepresentation analysis of the identified genes indicated that 5C cells respond to E_2 by suppressing ER α signaling and producing endoplasmic reticulum and inflammatory stress. Estrogen signaling was suppressed by metabolically reducing intracellular E_2 concentrations (increased CYP1B1 and HSD17B11) and up-regulating genes that antagonize ER α activity (SETD7, FHL2, Snail 1, COUP-TF1, TR α , AR, INHBA, and SMAD3) or repressing genes that promote ER α activity (AREG, PIK3CB, and CAV1). ERS was indicated by a deficiency in up-regulating genes involved in initiating a UPR (GRP78, XBP1, and S1P), protein

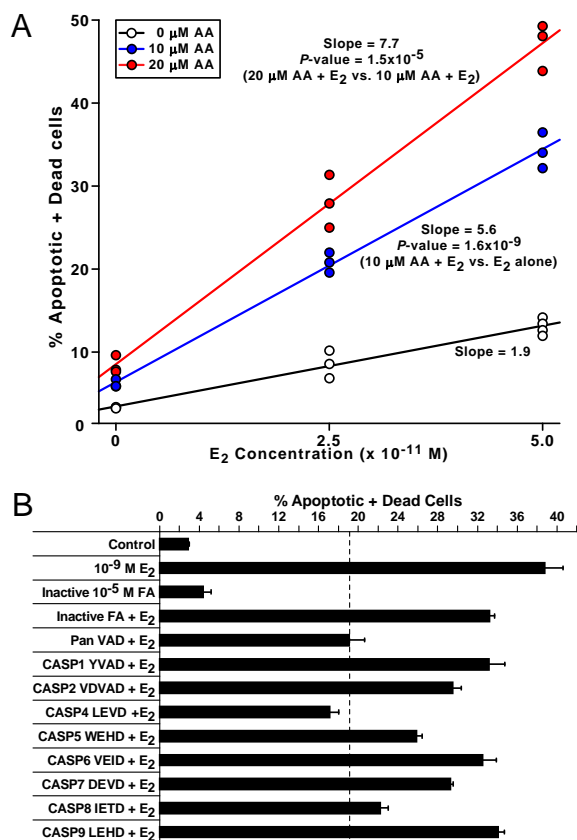


Fig. 4. Functional interrogation of E₂-induced apoptosis. (A) AA and E₂ interact to superadditively induce apoptosis. 5C cells were treated with combinations of AA and E₂ as indicated for 72 h. (B) Screening of selective CASP inhibitors. The selectivity of the inhibitors for individual caspases is indicated according to the manufacturer. 5C cells were treated with 10⁻⁹ M E₂ and 10 μM of each CASP inhibitor as indicated for 96 h. (A and B) Apoptosis according to altered plasma membrane permeability was determined by flow cytometric analysis of cells stained with the DNA-specific binding dyes YO-PRO-1 and 7-aminoactinomycin D. Double-negative staining cells were defined as viable, double-positive staining cells were defined as dead, and intermediately staining cells were defined as apoptotic. Data shown in B represent triplicates and associated SDs.

folding (GRP78, PDIA6, and UGGT1), and degradation of malformed proteins (HERP1 and DERL1), which would lead to accumulation of unfolded/misfolded proteins. Meanwhile, expression profiles indicated a widespread inhibition of protein translation (increased p58^{IPK} and decreased aminoacyl tRNA synthetases, EEF2K, and ERF3A) and fatty acid synthesis (decreased ACLY and SCD), which combined with accumulation of unfolded proteins, would also promote stress and apoptosis. ERS was also indicated by induction of BIM, BAX, and the inflammatory caspase CASP4. We previously showed that depletion of BIM or BAX blocked E₂-induced apoptosis (31), and here, we showed that blocking CASP4 with z-LEVD-fmk also blocked E₂-induced apoptosis. Inflammatory stress was indicated by up-regulation of cytokines/cytokine receptors (IL-4R, IL-6R, IL-6ST/gp130, IL-17RD, and LTB), IFN/IFN responsive genes (IFNL1, IFI6, and IFI16), AA biosynthetic genes (FADS1 and PLA2G10), and other inflammatory markers (CEBPB, NTN1, and UNC5C). These findings indicate that inflammatory and ERS responses leading to apoptosis are highly interrelated and may cross-talk in part through NF-κB, JNK, and AP-1. Thus, additional stimulation of ERS and inflammatory responses by AA interacted with E₂ to superadditively induce apoptosis.

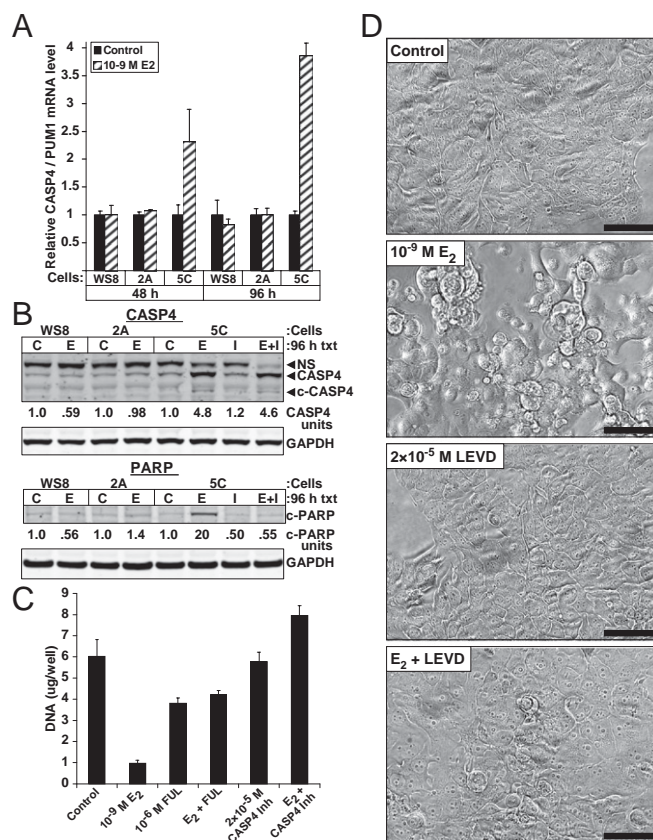


Fig. 5. Functional involvement of CASP4 in E₂-induced apoptosis. E₂-induced CASP4 at the (A) mRNA and (B) protein levels in 5C cells but not in WS8 or 2A cells. CASP4 mRNA and protein levels were measured by qPCR and immunoblotting, respectively. (B) In 5C cells, E₂ led to cleavage of the apoptotic marker PARP, which was blocked by the CASP4 inhibitor z-LEVD-fmk. C, control; E, E₂; I, inhibitor (z-LEVD-fmk); c-CASP4, cleaved CASP4; NS, non-specific band. (C) E₂-inhibited growth of 5C cells was completely reversed by z-LEVD-fmk. Proliferation was determined after 6 d of 10⁻⁹ M E₂ exposure and measured by DNA mass per well. CASP4 inh, CASP4 inhibitor z-LEVD-fmk. (D) Morphologic alterations after 96 h of 10⁻⁹ M E₂ in 5C cells were completely reversed by z-LEVD-fmk (LEVD). (Scale bar: 100 microns.) (A–D) E₂ was used at 10⁻⁹ M and z-LEVD-fmk at 2 × 10⁻⁵ M. Data in A and C represent the average and SDs of four and eight replicates, respectively.

It should be noted that the differentially expressed genes identified here are associated with E₂-induced apoptosis; hence, their causal role in apoptosis needs to be functionally validated. Additional characterization and functional validation of genes and pathways regulating E₂-mediated apoptosis in 5C cells is currently being investigated using genome-wide, high-throughput RNAi profiling. Also, the results presented here are based on only MCF-7 derivative cell lines; hence, the findings may have limited applicability to the clinic. However, the MCF-7 cell line has accurately predicted clinical responses to antihormonal therapy in breast cancer (35). We and others have reported antihormonal-resistant MCF-7-based models besides 5C cells that exhibit E₂-induced apoptosis in vitro and in vivo (18, 19, 23, 36). We are aware of only one other breast cancer model not derived from MCF-7 cells that exhibits this behavior (i.e., T47D cells stably expressing PKCα), but only when grown in vivo as xenograft tumors (37). Therefore, molecular markers of ERS and inflammatory stress need to be confirmed in low-dose E₂ responding compared with nonresponding tumors in patients with estrogen-deprived metastatic breast cancer.

The identified 5C-specific genes may serve as biomarkers to predict response to estrogen therapy (e.g., the secreted factors

IFNL1, LTB, and NTN1 could be readily measured in patients). The identified 5C-specific genes also provide the basis for potentially improving clinical response rates to estrogen by combining it with agents that promote ERS and/or tumor-specific inflammation. For example, neutralizing NTN1 antibodies, AA, or its precursor, conjugated LA (Dataset S5, ref. 51), may increase response rates without engaging systemic inflammatory responses. Furthermore, these findings lead to the hypothesis that antiinflammatory agents prescribed for ancillary clinical problems should not be used during antitumor estrogen therapy.

Methods

Generation and Validation of RNA Samples for Microarrays. Each cell line was treated with or without 10^{-9} M E_2 using six replicates per treatment for 2, 6, 12, 24, 48, 72, and 96 h. To validate that each isolated RNA sample was derived from cells appropriately treated with or without E_2 , expression of two classical E_2 -responsive genes, MYC and TFF1 (pS2), were measured using real-time qPCR. MYC exhibited early kinetics, and TFF1 exhibited later kinetics of E_2 induction; together, induction of these markers spanned the entire time course. Successfully validated samples are shown in Figs. S6–S8.

dAUC Analysis. Differentially labeled fluorescent cRNA probes for each individual E_2 -treated RNA sample (Cy3) and time point-matched, pooled, control-treated RNA samples (Cy5) were competitively hybridized to Agilent 4 × 44 K oligonucleotide microarrays using standard Agilent protocols. Gene expression values were extracted from arrays as relative \log_2 ratios of E_2 /control-treated cells. To determine whether a gene's regulation by E_2 was significantly different between two cell lines, a method termed dAUC

analysis was developed. In this method, the quantity of interest (dAUC) for a given probe is calculated as the signed area between the expression profiles for the two cell lines (using the average observed values at each time point). The null hypothesis is that dAUC is zero, and the distribution of dAUC values under the null hypothesis can be obtained by repeatedly permuting ($n = 20,000$) the cell line to which each \log_2 ratio value was assigned, while keeping the time points fixed. The two-sided P value of the observed dAUC can then be calculated as the proportion of permutations yielding a dAUC that exceeds the observed dAUC in absolute value. A probe was considered significantly different between two cell lines if the magnitude of the observed dAUC exceeded that obtained in all permutations (i.e., $P < 0.00005$). To exclude probes with statistically significant but numerically small differences, we imposed an additional condition that the probe's dAUC must have exhibited an average \log_2 fold change of 0.58 (1.5-fold on a linear scale) across a given time period. The dAUCs of each probe were calculated using all pairwise combinations of the three cell lines over the entire 2–96 h time course, and to delineate relatively early and late response genes, they were calculated over 2–24 and 24–96 h time periods.

ACKNOWLEDGMENTS. We thank Olga Tchuvatkina for programming a sample tracking system. We thank Michael Bittner, Seungchan Kim, Samuel Litwin, Suraj Peri, Karthik Devarajan, and Yan Zhou for assistance with bioinformatic analyses. We thank John Taylor for technical assistance. This work was supported by Department of Defense Center of Excellence Grant W81XWH-06-1-0590 (to V.C.J.) and Weg and Genuardis funds (to V.C.J.) and National Institutes of Health Grants P30CA006927 (to Fox Chase Cancer Center) and P30CA051008 (to Lombardi Comprehensive Cancer Center, Georgetown University).

- Dodds EC, Goldberg L, Lawson W, Robinson R (1938) Estrogenic activity of certain synthetic compounds. *Nature* 141:247–248.
- Robson JM, Schönberg A (1937) Oestrous reactions including mating produced by triphenylethylene. *Nature* 140:196.
- Haddow A, Watkinson JM, Paterson E, Koller PC (1944) Influence of synthetic oestrogens on advanced malignant disease. *Br Med J* 2:393–398.
- Haddow A (1970) David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26:737–754.
- Lerner LJ, Jordan VC (1990) Development of antiestrogens and their use in breast cancer: Eighth Cain memorial award lecture. *Cancer Res* 50:4177–4189.
- Jordan VC (2003) Tamoxifen: A most unlikely pioneering medicine. *Nat Rev Drug Discov* 2:205–213.
- Jordan VC (2008) Tamoxifen: Catalyst for the change to targeted therapy. *Eur J Cancer* 44:30–38.
- Jordan VC (2001) Selective estrogen receptor modulation: A personal perspective. *Cancer Res* 61:5683–5687.
- Jordan VC, Phelps E, Lindgren JU (1987) Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10:31–35.
- Cummings SR, et al. (1999) The effect of raloxifene on risk of breast cancer in postmenopausal women: Results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281:2189–2197.
- Vogel VG, et al. (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295:2727–2741.
- Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: An overview of the randomised trials. *Lancet* 351:1451–1467.
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 365:1687–1717.
- Berry DA, et al. (2006) Modeling the impact of treatment and screening on U.S. breast cancer mortality: A Bayesian approach. *J Natl Cancer Inst Monogr* 36:30–36.
- Dowsett M, et al. (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* 28:509–518.
- Osborne CK, Coronado EB, Robinson JP (1987) Human breast cancer in the athymic nude mouse: Cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 23:1189–1196.
- Gottardis MM, Jordan VC (1988) Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48:5183–5187.
- Wolf DM, Jordan VC (1993) A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 127:23–33.
- Yao K, et al. (2000) Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6:2028–2036.
- Jordan VC (2004) Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell* 5:207–213.
- Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y (1987) Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 47:4355–4360.
- Welshons WV, Jordan VC (1987) Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer Clin Oncol* 23:1935–1939.
- Song RX, et al. (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 β -estradiol. *J Natl Cancer Inst* 93:1714–1723.
- Lewis JS, et al. (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97:1746–1759.
- Lewis-Wambi JS, et al. (2008) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 10:R104.
- Lonning PE, et al. (2001) High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 67:111–116.
- Lonning PE (2009) Additive endocrine therapy for advanced breast cancer—back to the future. *Acta Oncol* 48:1092–1101.
- Ellis MJ, et al. (2009) Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: A phase 2 randomized study. *JAMA* 302:774–780.
- Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 90:77–86.
- Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 55:2583–2590.
- Pink JJ, Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56:2321–2330.
- Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94:131–141.
- Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, Jordan VC (2008) Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. *Eur J Cancer* 44:1770–1779.
- Hu ZZ, et al. (2011) Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One* 6:e20410.
- Levenson AS, Jordan VC (1997) MCF-7: The first hormone-responsive breast cancer cell line. *Cancer Res* 57:3071–3078.
- Liu H, et al. (2003) Apoptotic action of 17 β -estradiol in raloxifene-resistant MCF-7 cells *in vitro* and *in vivo*. *J Natl Cancer Inst* 95:1586–1597.
- Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC, Tonetti DA (2001) Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase Alpha. *Clin Cancer Res* 7:3156–3165.

Supporting Information

Ariazi et al. 10.1073/pnas.1115188108

SI Results and Discussion

Cell Line Characterization. The expected growth/apoptotic responses to 17 β -estradiol (E_2), biomarker statuses of estrogen receptor- α (ER α), PgR, and HER2, and ER-regulated transcriptional activity were confirmed in WS8, 2A, and 5C cells (Fig. S1). Estrogen-dependent WS8 cells exhibited a 6.8-fold increase in growth after 7 d of 10^{-9} M E_2 treatment compared with control (no E_2) treatment (Fig. S1A). Estrogen deprivation-resistant 2A cells grew robustly in the absence of E_2 over 12 d. E_2 did not affect growth of the 2A cells over the first 6 d but did inhibit growth beginning at day 7 to day 12 by 62.5% (Fig. S1B). Hence, the 2A cells exhibited an initial phase of E_2 -independent growth followed by a second phase of E_2 -inhibited growth. The resistant 5C cells continually proliferated in the absence of E_2 over 7 d. The DNA mass per well of the 5C cells also increased in the presence of E_2 but only for the first 4 d. It is important to note that, within this period of apparent growth, E_2 caused subtle morphologic changes by day 2 and gross morphologic changes in the 5C cells, such as rounding, blebbing, and detachment from the plate, by day 4 (Fig. S1D). In E_2 -treated 5C cells, DNA mass per well steadily decreased from day 4 to day 7 such that, by day 7, there was 5% less DNA than at day 1 of the experiment (Fig. S1C). Therefore, the 5C cells displayed a relatively rapid E_2 -induced growth inhibitory response compared with the delayed growth inhibitory response in the 2A cells. We previously reported that these growth inhibitory responses to E_2 in 5C cells reflect induction of apoptosis (1).

Protein levels of ER α , PgR, and HER2 were characterized by semiquantitative immunoblot analysis in estrogen-dependent WS8 and estrogen deprivation (ED) resistant 2A and 5C cells (Fig. S1D). Both 2A and 5C cells overexpressed ER α : 2A cells by 5.8-fold and 5C cells by 2.3-fold compared with WS8 cells. Incubation of the cells with 10^{-9} M E_2 for 48 h decreased ER α protein levels and induced PgR protein in WS8 and 2A cells but not 5C cells. Thus, WS8 and 2A cells were PgR-positive (2), whereas 5C cells were PgR-negative as previously reported (3). HER2 protein levels in control-treated cells were not substantially different in both 2A and 5C cells compared to WS8 cells after correction for the loading control β -actin. Therefore, HER2 was unlikely to have contributed to the development of ED resistance in these cells.

ER transcriptional activity was evaluated using an estrogen responsive element (ERE)-regulated dual luciferase reporter gene system. Cells were transfected and treated with increasing concentrations of E_2 for 24 h (Fig. S1E). Basal (control treatment) ERE-dependent transcriptional activity was 5.8-fold in 2A cells ($P < 0.0001$), and 1.7-fold in 5C cells ($P = 0.001$) relative to control-treated WS8 cells (Fig. S1E). Hence, unliganded ER transcriptional activity was higher in 2A and 5C cells and correlated with increased ER α protein levels (Fig. S1D). E_2 at 10^{-9} M stimulated ER-dependent transcriptional activity in both ED-resistant cell lines: to a greater extent in resistant 2A cells (22.5-fold) than estrogen-dependent WS8 cells (15.4-fold) and to a lesser extent in resistant 5C cells (7.4-fold) in which E_2 induces apoptosis.

Characterization of E_2 -Induced Apoptosis. Apoptosis was verified in 5C cells in response to E_2 based on loss of plasma membrane integrity. This verification was determined by flow cytometric analysis of cells stained with the DNA binding dyes YO-PR0-1 and 7-aminoactinomycin D. Viable cells excluded these dyes, whereas apoptotic cells allowed moderate staining; dead cells

stained strongly. First, the concentration response of E_2 -induced apoptosis was assessed (Fig. S2A and B). 5C cells were treated with increasing concentrations of E_2 from 10^{-11} to 10^{-8} M or control for 96 h. An apoptotic response was detected with just 10^{-11} M E_2 (8.0% vs. 1.9% in control-treated cells), the lowest concentration of E_2 tested. Full apoptotic responses occurred at and above 10^{-10} M E_2 (37.2%), with a maximal response at 10^{-8} M E_2 (44.9%). Fitting a sigmoidal dose response curve (four-point logistic equation) to the data revealed that the EC_{50} of E_2 -induced apoptosis was 3.0×10^{-11} M. Second, the dependency of the apoptotic response on ER was confirmed. In addition to E_2 , the synthetic estrogen diethylstilbestrol (DES) at 10^{-9} M stimulated a robust apoptotic response, and importantly, the pure antiestrogen fulvestrant completely blocked both E_2 - and DES-induced apoptosis (Fig. S2C). Therefore, ER mediated E_2 -induced apoptosis.

SI Methods

Cell Lines and Compounds. MCF-7:WS8 human breast cancer cells were clonally selected from MCF-7 cells for sensitivity to E_2 -stimulated growth (2–4) and used here as the estrogen-dependent reference cell line. ED-resistant MCF-7:2A (2, 4, 5) and MCF-7:5C (1, 3, 6) human breast cancer cells were also clonally selected from MCF-7 cells for maximal growth under long-term estrogen-free conditions. Estrogen-dependent WS8 cells were maintained in fully estrogenized media (phenol red containing RPMI-1640 and 10% whole FBS supplemented with 6 ng/mL insulin, 2 mM glutamine, 100 μ M nonessential amino acids, and 100 U penicillin and streptomycin per mL), whereas 5C and 2A cells were maintained in estrogen-free medium (phenol red-free RPMI-1640 plus 10% dextran-coated charcoal-stripped FBS and the same supplements as for fully estrogenized medium) as previously described. Cells were maintained at 37 $^{\circ}$ C in a humidified 5% CO_2 atmosphere. Estrogen-dependent WS8 cells were switched to estrogen-free media for 3 d before all experiments. E_2 and DES were from Sigma-Aldrich. Fulvestrant (also termed ICI 182,780 and Faslodex) was from Tocris. All cell culture reagents were from Invitrogen. Caspase substrate peptide inhibitors of the generalized sequence z-XXXX-fmk (z, benzyloxycarbonyl; X, any amino acid; fmk, fluoromethyl ketone) were from Biovision. The peptides are derivatized as methyl esters to promote cell permeability and to fmk to irreversibly inhibit the caspase by alkylating a cysteine residue in the catalytic site. All test agents were added to culture medium at 1:10,000–1:1,000 (vol/vol).

Cellular Proliferation. Cellular proliferation was assessed according to DNA mass per well using Hoechst 33258 (Invitrogen) as previously described (1). WS8 and 5C cells were seeded at 15,000 and 20,000 cells/well, respectively, in 24-well plates and allowed to grow for 7 d. 2A cells were seeded at 30,000 cells/well in six-well plates and allowed to grow for 12 d. Cells were treated without (control) or with 10^{-9} M E_2 every other day.

Immunoblot Analyses. Whole-cell protein lysates were prepared and immunoblotted using 40 μ g protein per lane as previously described (7). Membranes were probed using antibodies against ER α (AER6111; Lab Vision), HER2 (EP1045Y; Epitomics), PgR (YR85; Epitomics), CASP4 (CAS4; Sigma-Aldrich), PARP (46D11; Cell Signaling Technology), β -actin (AC-15; Sigma-Aldrich), and GAPDH (14C10; Cell Signaling Technology). Blots were visualized and quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Protein units in figures re-

flect the relative level of the target protein normalized to the endogenous control protein.

ERE Dual-Luciferase Assays. ERE dual-luciferase assays were conducted by transfecting cells with an ERE (5 \times)-regulated [pERE (5 \times) TA-ffLuc] firefly luciferase expression plasmid, and co-transfected with a basal TATA promoter-regulated (pTA-srLuc) *Renilla* luciferase expression plasmid as previously described (7).

Apoptosis Analysis by Cell Membrane Permeability Assay. The percentage of apoptotic cells was determined based on altered plasma membrane permeability to the nucleic acid stains YO-PRO-1 and 7-aminoactinomycin D and analyzed by flow cytometry. Using these dyes, viable cells stain weakly, apoptotic cells stain moderately, and dead cells stain strongly. Adherent cells were harvested by trypsinization and combined with floating cells. Cells were suspended to $\sim 500,000$ cells/500 μ L in estrogen-free media and incubated with 100 nM YO-PRO-1 (Invitrogen) plus 1 μ g/mL 7-aminoactinomycin D (Invitrogen) at 37 $^{\circ}$ C for 60 min. Immediately afterward, cells were kept on ice and then analyzed using a BD LSR-II flow cytometer (BD Biosciences). At least 30,000 singlet events were collected per sample. Stains were excited using a 488-nm laser and detected using 530- (YO-PRO-1) and 670-nm (7-aminoactinomycin D) bandpass filters. Spectral compensation between dyes was accomplished using single-stained cells. Sequential gating of the relevant forward scatter area vs. forward scatter height and forward scatter height vs. side scatter area population subsets allowed for the selection of single cells. Data were analyzed using FloJo 7.6.1 for Windows (Tree Star).

RNA Sample Generation for Microarray Analysis. WS8, 2A, and 5C cells were seeded at 2, 4, and 5 million cells/15-cm plate, respectively, in estrogen-free media. Cells were parsed into two groups of six replicate plates per treatment per time point and then treated with either 0.1% ethanol (vehicle control) or 10^{-9} M E_2 for 2, 6, 12, 24, 48, 72, and 96 h. At 48 h, media on the remaining cells were replenished. Cells were harvested for RNA using TRIzol. In total, 252 samples were collected. Total RNA was isolated as previously described (8). RNA samples were controlled for purity and integrity using a Nanodrop 1,000 spectrophotometer and an Agilent 2100 Bioanalyzer by requiring each sample to exhibit an RNA integrity number of 9.8–10.0.

Real-Time Quantitative PCR Assays. Real-time quantitative PCR (qPCR) was conducted as previously described (7). Target mRNA levels were normalized to pumilio homolog 1 (*Drosophila*) mRNA levels (9). Data were analyzed by comparison with a serial dilution series of WS8 cell cDNA. PCR primer sequences were as follows: PUM1 forward 5'-AAT GCA GGC GCG AGA AAT-3', PUM1 reverse 5'-TTG TGC AGC TGA GGA ACT AAT GA-3', PUM1 probe 5'-[6FAM]-CCT GTT CGA CTT GTA GCT CCT GCC CC-[BHQ1]-3'; MYC forward 5'-GCC ACG TCT CCA CAC ATC AG-3', MYC reverse 5'-TCT TGG CAG CAG GAT AGT CCT T-3', MYC probe 5'-[6FAM]-ACG CAG CGC CTC CCT CCA CTC-[BHQ1]-3'; TFF1 forward 5'-CAT CGA CGT CCC TCC AGA AGA G-3', TFF1 reverse 5'-CTC TGG GAC TAA TCA CCG TGC TG-3', (no probe for TFF1); CASP4 forward 5'-TTT CCT GGC AAT TGA AAA TGG-3', CASP4 reverse 5'-AAG GTG CTC CTT GAA GTT GAT TAA G-3', CASP4 probe 5'-[6FAM]-AGC CAC AAG CAG CCC AGC CCT-[BHQ1]-3'; FOS forward 5'-GCG TTG TGA AGA CCA TGA CA-3', FOS reverse 5'-CCT TCG GAT TCT CCT TTT CTC T-3', FOS probe 5'-[6FAM]-AGG CCG AGC GCA GAG CAT TG-3-[BHQ1]-3'. All other qPCR primers/probes were predesigned assays from Life Technologies/Applied Biosystems.

Gene Expression Microarrays. Gene expression profiling was carried out using two-color Agilent 4 \times 44 K Whole Human Genome oligonucleotide microarrays. RNA labeling, hybridization to the arrays, and quality assessment of hybridizations were performed using protocols recommended by Agilent Technologies as previously described (8). Each individual E_2 -treated RNA sample was competitively hybridized against a time point-matched, control-treated reference RNA, which consisted of a pool of equal amounts of RNA from six replicate control-treated samples (in some cases, five samples if a sample could not be validated). Individual E_2 -treated RNA samples were labeled with Cy3, and control-treated RNA reference pools were labeled using Cy5. All replicate Cy3 samples were competitively hybridized against the same reference pool within each time point. Raw data were extracted, processed, and normalized using Agilent's Feature Extraction software (v10.7) as previously described (8). After applying a set of array hybridization QC criteria (8) and estrogen-responsive marker qPCR analysis, eight arrays were excluded from additional analysis—as a result, 4 of 21 (cell line/time point) combinations had five replicate arrays, 3 combinations had four replicate arrays, and the remaining 16 combinations had six replicate arrays each; in total, there were 118 arrays. The gene expression data are publically available online [Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE29917)].

Several steps were taken to minimize variability across this large gene expression microarray series. The cell line treatment series was conducted consecutively (WS8 followed by 5C and 2A). Within each cell line series, the same lot numbers of Qiagen RNeasy kits, Agilent arrays, gasket backings, Cy-dyes labeling kits, hybridization kits, and wash buffers were used. All arrays were washed in an ozone-controlled environment (<0.1 ppb). Samples representing replicate (cell line per time point) assays were hybridized to different arrays to minimize any chip to chip variation across replicates.

Differential Area Under the Curve Analysis. The expression measurements used in all analyses were the \log_2 ratio values produced by Agilent's Feature Extraction software. Before analysis, identical probes appearing on multiple spots were replaced by their first occurrence, and probes lacking a valid Entrez identifier (based on Bioconductor annotation) were omitted. Probes that were absent (those with signals indistinguishable from background as defined by Feature Extraction software) in both channels across all arrays involved in the comparison of interest were removed. Probes that were flagged by Feature Extraction as nonuniformity outliers in any of the replicate arrays were also removed.

For a given cell line (WS8, 2A, or 5C) and probe, a measure of the effect of E_2 treatment over all or part of the time course is given by the area under the \log_2 ratio profile over the time period of interest. Let x_{ink} be the observed \log_2 ratio for probe p for cell line i , time t_n ($t_1 = 2, \dots, t_7 = 96$), and replicate k [where k ranges from 1 to the total number of replicate arrays for cell line i and time t_n (six in most cases)], and let x_{in} be the average \log_2 ratio across replicates. Then, the area under the \log_2 ratio profile (AUC_{*i*}) can be calculated as a sum of trapezoidal areas (Eq. S1):

$$AUC_i = \sum_{n=1}^6 \frac{(t_{n+1} - t_n)(x_{in} + x_{i(n+1)})}{2} \quad [S1]$$

This area can be positive or negative, reflecting, respectively, a net up- or down-regulation with E_2 treatment across the time course. Differential AUC (dAUC_{*ij*}) = AUC_{*i*} – AUC_{*j*} provides a measure of change in regulation between two cell lines i and j across a time interval. To assess the statistical significance of this difference, we generate a reference distribution (for each probe)

for $dAUC_{ij}$ under the null hypothesis that the true differential AUC is zero; we repeatedly ($n = 20,000$) permuted the cell line labels within each time point and calculated $dAUC_{perm}$ for each permutation. The two-sided P value of the test is the proportion of permutations for which the absolute value of the calculated $dAUC_{perm}$ exceeds the absolute value of the observed $dAUC_{ij}$. Only those probes whose observed $dAUC_{ij}$ value exceeded all 20,000 resampled $dAUC_{perm}$ values (i.e., P value < 0.00005) were considered significant. To select only those probes exhibiting a substantial separation between expression profiles, differential AUCs were also required to exhibit an average \log_2 fold difference of at least 0.58 (corresponding to a 1.5-fold change on the linear scale)—the average \log_2 fold difference across a time course is the ratio of $dAUC_{ij}$ to the length of the time course. All three pairwise comparisons between the three cell lines were performed. The AUC statistics for the full time course and the early ($t = 2, 6, 12$, and 24 h) and late ($t = 24, 48, 72$, and 96 h) subsets of the time course were computed. Finally, if multiple distinct probes mapped to the same Entrez gene, only the probe exhibiting the greatest overall expression intensity, measured by the sum of the \log_2 intensities in both channels across all arrays in the comparison, was retained. All identified 5C-specific genes are listed in Dataset S1 with full annotation, $dAUC$ values, and P values.

In some instances, it was necessary to assess differential expression at a single time point. For these comparisons, we used the *limma* package (10, 11) implemented in the R/Bioconductor platform (12). *limma* was used to compute empirical Bayes-moderated t statistics, analogous to classical t statistics except that information on all probes is used to produce more stable SE estimates of log fold changes. P values of moderated t statistics

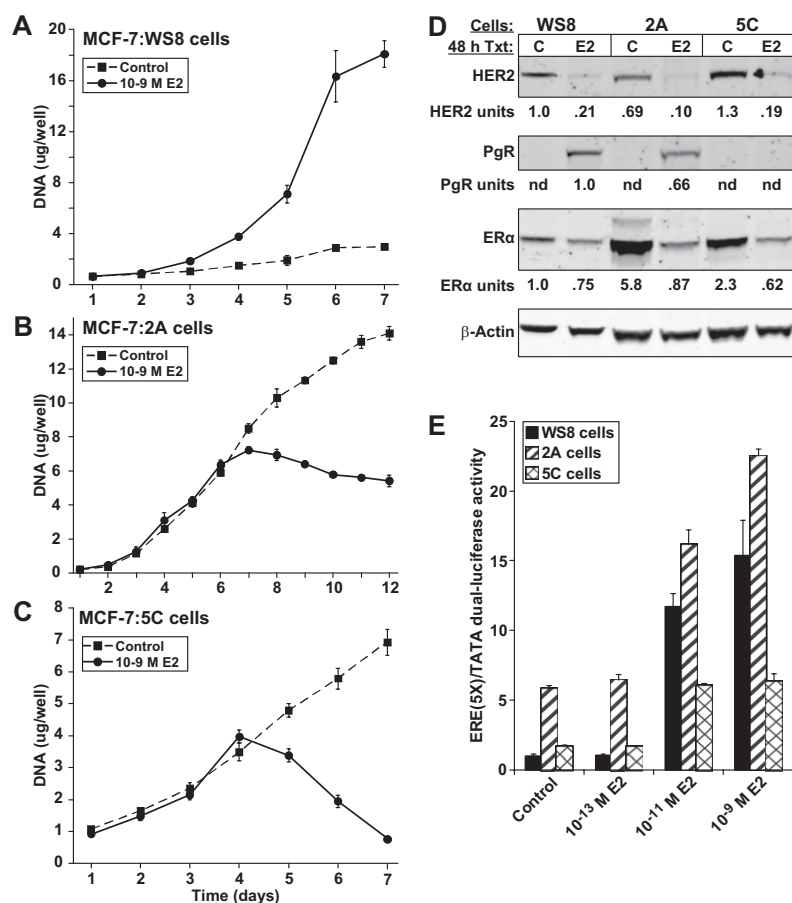
were adjusted for multiple comparisons by using the method of Benjamini and Hochberg (13) to control the false discovery rate, the expected false positive rate among rejected null hypotheses.

Gene Enrichment and Pathway Analysis. Gene enrichment and pathway analysis (Fig. S3) was conducted using GeneGo's MetaCore version 6.5. This software generates P values based on a hypergeometric test of enrichment and measures the probability of observing the number of identified genes mapping to a particular curated process by chance as a function of the total number of identified genes, the number of curated genes in the pathway, and the size of the full set of all genes in all curated pathways. Significantly enriched processes/pathways/networks were required to pass a false discovery rate of 0.05.

Genes differentially regulated by E_2 selectively in 5C cells that are involved in estrogen signaling, apoptosis, and inflammatory responses are listed in Datasets S2–S4, respectively. These genes were categorized according to gene ontology terms as curated by GeneGo and the Gene Ontology project (<http://geneontology.org>).

Additional Statistical Analyses. Two-sided t tests were used for pairwise comparisons not involving microarray gene expression values. Nonlinear curve fitting and EC_{50} determinations were performed using Prism 4.03 (GraphPad Software). To assess the interaction between E_2 - and arachidonic acid (AA)-induced apoptosis (Fig. 4A), the multiple regression model $Pct = E_2 + AA + E_2 \times AA$ was fit, in which Pct was the percentage of apoptotic plus dead cells, E_2 was the E_2 concentration, and AA was the AA concentration. AA was coded as a categorical factor with three levels (0, 10, and 20 μM).

- Lewis JS, et al. (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97:1746–1759.
- Pink JJ, Jiang SY, Fritsch M, Jordan VC (1995) An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 55:2583–2590.
- Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC (1992) An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 90:77–86.
- Pink JJ, Jordan VC (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56:2321–2330.
- Lewis-Wambi JS, et al. (2008) Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 10:R104.
- Lewis JS, Osipo C, Meeke K, Jordan VC (2005) Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94:131–141.
- Ariazi EA, Kraus RJ, Farrell ML, Jordan VC, Mertz JE (2007) Estrogen-related receptor $\alpha 1$ transcriptional activities are regulated in part via the ErbB2/HER2 signaling pathway. *Mol Cancer Res* 5:71–85.
- Willis AL, et al. (2008) The fibroblast growth factor-inducible 14 receptor is highly expressed in HER2-positive breast tumors and regulates breast cancer cell invasive capacity. *Mol Cancer Res* 6:725–734.
- Lyng MB, Laenkholm AV, Pallisgaard N, Ditzel HJ (2008) Identification of genes for normalization of real-time RT-PCR data in breast carcinomas. *BMC Cancer* 8:20.
- Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3:Article 3.
- Smyth GK (2005) Limma: Linear Models for Microarray Data. *Bioinformatics and Computational Biology Solutions Using R and Bioconductor, Statistics for Biology and Health*, eds Gentleman RC, Carey VJ, Huber W, Irizarry RA, Dudoit S (Springer, New York), pp 397–420.
- Gentleman RC, et al. (2004) Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol* 5:R80.
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Statist Soc B* 57:289–300.



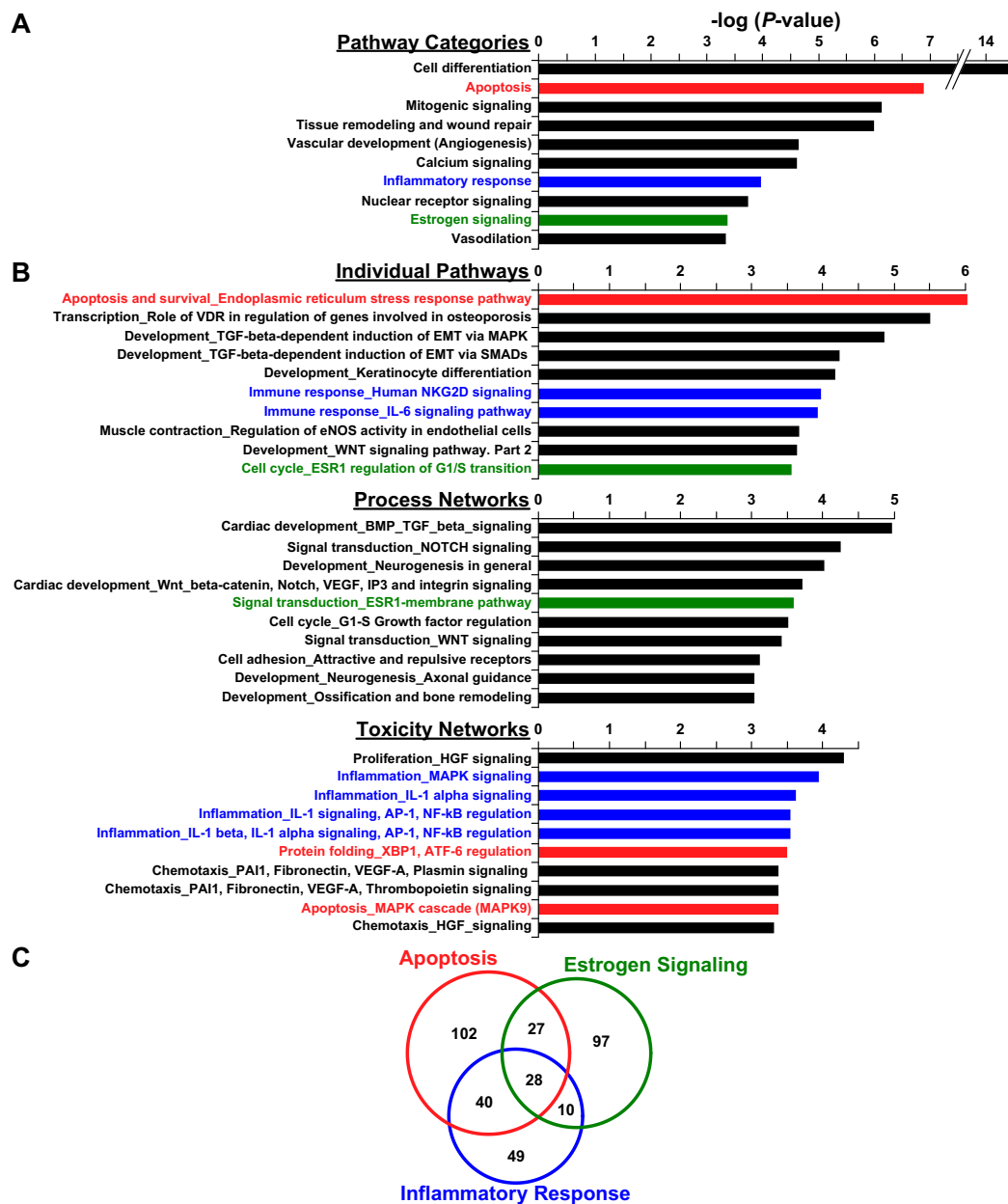


Fig. S3. Enrichment analysis of genes differentially regulated by E₂ in 5C cells relative to both WS8 and 5C cells. (A) Broad pathway categories. (B) Individual pathways and networks. (A and B) The top 10 scoring pathways/processes are shown for each group. (C) Venn diagram showing the total and overlapping number of genes involved in estrogen signaling, inflammatory response, and apoptosis. (A–C) Estrogen signaling pathways are shown in green, apoptosis-related pathways are shown in red, and inflammatory pathways are shown in blue.

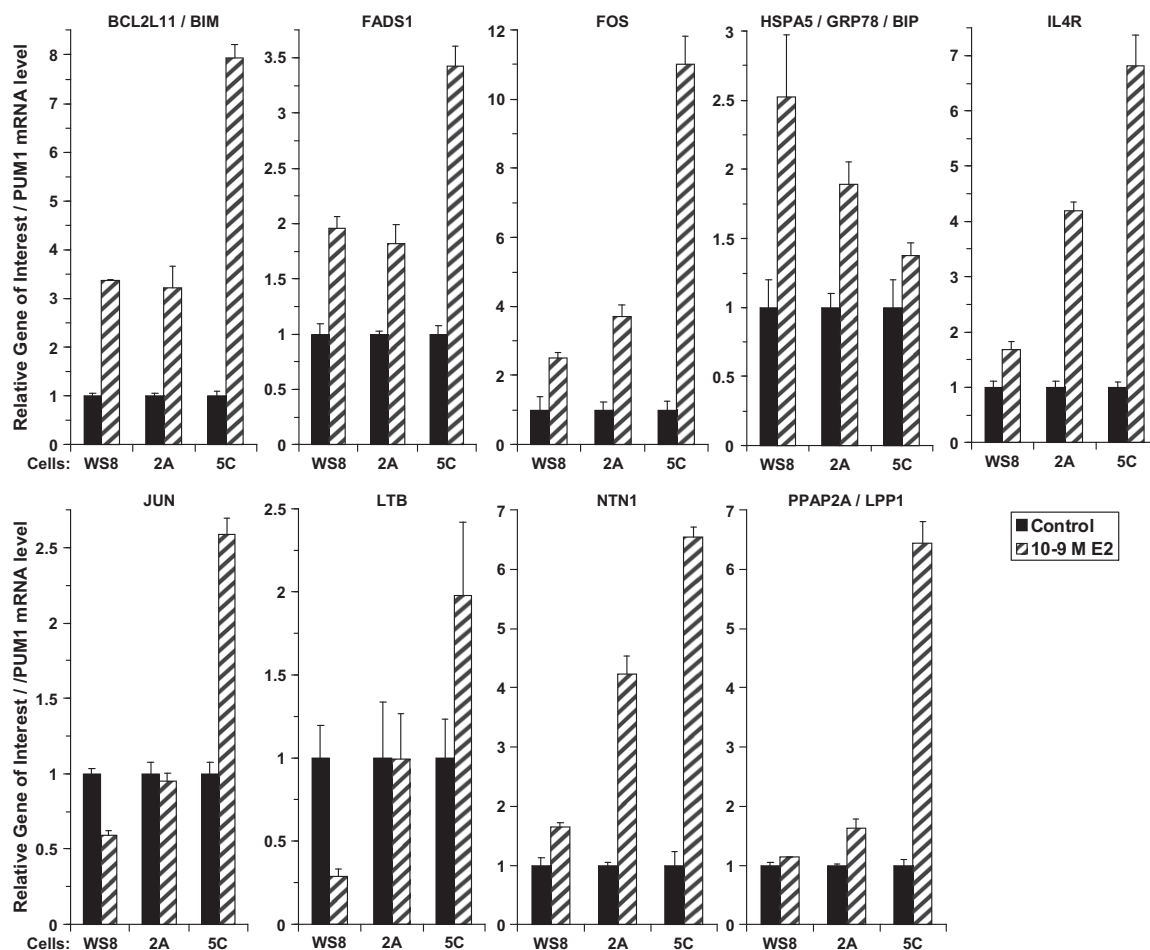


Fig. S4. Genes verified by qPCR to show differential expression in 5C cells compared with WS8 and 2A cells. Cells were treated with or without 10^{-9} M E_2 for 72 h. RNA expression levels of the indicated genes were determined by real-time qPCR. Data shown represent quadruplicates and associated SDs.

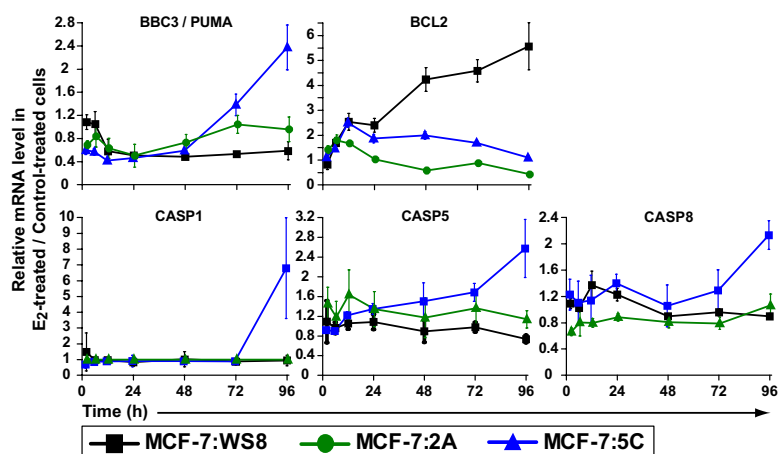


Fig. S5. Discussed apoptotic genes that did not meet the requirements for significance as differentially expressed over time in 5C cells compared to WS8 and 2A cells.

Four decades of discovery in breast cancer research and treatment – an interview with V. Craig Jordan

MARC POIROT*

Directeur de Recherche à l'INSERM, INSERM UMR 1037, University of Toulouse III, Toulouse, France

ABSTRACT V. Craig Jordan is a pioneer in the molecular pharmacology and therapeutics of breast cancer. As a teenager, he wanted to develop drugs to treat cancer, but at the time in the 1960s, this was unfashionable. Nevertheless, he saw an opportunity and through his mentors, trained himself to re-invent a failed “morning-after pill” to become tamoxifen, the gold standard for the treatment and prevention of breast cancer. It is estimated that at least a million women worldwide are alive today because of the clinical application of Jordan’s laboratory research. Throughout his career, he has always looked at “the good, the bad and the ugly” of tamoxifen. He was the first to raise concerns about the possibility of tamoxifen increasing endometrial cancer. He described selective estrogen receptor modulation (SERM) and he was the first to describe both the bone protective effects and the breast chemopreventive effects of raloxifene. Raloxifene did not increase endometrial cancer and is now used to prevent breast cancer and osteoporosis. The scientific strategy he introduced of using long term therapy for treatment and prevention caused him to study acquired drug resistance to SERMs. He made the paradoxical discovery that physiological estrogen can be used to treat and to prevent breast cancer once exhaustive antihormone resistance develops. His philosophy for his four decades of discovery has been to use the conversation between the laboratory and the clinic to improve women’s health.

KEY WORDS: *tamoxifen, raloxifene, acquired antihormone resistance, estrogen, nonsteroidal antiestrogen, selective estrogen receptor modulator (SERM), estradiol-induced apoptosis*

The past is never dead. It is not even the past.
William Faulkner

Tamoxifen, originally classified as a nonsteroidal antiestrogen but now known as the first selective estrogen receptor modulator (SERM), is a pioneering medicine that for more than twenty years was the gold standard for the adjuvant treatment of breast cancer in pre and postmenopausal patients with estrogen receptor (ER)-positive tumors (Jordan, 2003). Millions of women continue to live longer and healthier lives because of tamoxifen treatment. Tamoxifen is also a pioneering medicine, as it is the first drug to be approved in the United States of America by the Food & Drug Administration (FDA) for the reduction of the incidence of breast cancer in high risk pre and postmenopausal women (Jordan, 2007).

Craig Jordan grew up with a passion for chemistry, but was specifically intrigued by the prospect of using organic chemistry to design drugs to treat cancer. At the age of thirteen, his mother allowed him to convert his bedroom into a chemistry laboratory,

where he often got into difficulties during his experiments, either setting the curtains on fire as a rather over reactive experiment was being thrown out of the window, or destroying the lawn outside. However, he did convince his mother that by using the chemistry of fertilizers, he could re-grow the lawn again, but when he did, it came out an interesting shade of blue! Craig had a passion for teaching, and the chemistry and biology teachers at his school, Moseley Hall Grammar School in Cheadle, Cheshire, England allowed him to have a laboratory to teach biochemistry. It was these same teachers who convinced his parents that he should apply

Abbreviations used in this paper: AACR, American Association for Cancer Research; ASCO, American Society of Clinical Oncology; CEE, conjugated equine estrogen, DES, diethylstilbestrol; DMBA, dimethylbenzanthracene; EBCTCG, Early Breast Cancer Trialists’ Collaborative Group; ECOG, Eastern Cooperative Oncology Group; ER, estrogen receptor; FDA, Food & Drug Administration; ICI, Imperial Chemical Industries; SERM, selective estrogen receptor modulator; STAR, Study of Tamoxifen and Raloxifene; TGF α , transforming growth factor alpha; WFEB, Worcester Foundation for Experimental Biology; WHI, Women’s Health Initiative.

*Address correspondence to: Marc Poirot. Directeur de Recherche à l’Inserm, Inserm UMR 1037, University of Toulouse III, Cancer Research Center of Toulouse, Institut Claudius Regaud, 20, rue du pont Saint Pierre, 31052 Toulouse Cedex, France. Fax: +335-6142-4631. e-mail: marc.poirot@inserm.fr

Final, author-corrected PDF published online: 14 October 2011.



Fig. 1. Before the ceremony for the degree of Doctor of Medicine *honoris causa* at Leeds University on the 18th of July, 2001. Dr. Edward R. Clark, my PhD supervisor (1969-1972) (left) and Dr. Ronnie Kaye, Head of my degree course (1965-1969) (center), formally from the Department of Pharmacology, University of Leeds, England. I am on the right side with my signature glass of Burgundy.

to university. By contrast, Craig was more content with the idea of becoming an organic chemistry technician at the research laboratories of Imperial Chemical Industries (ICI) near where he lived.

Craig was given an opportunity for interview at only one university (Leeds University, West Yorkshire, England), but he succeeded in convincing the two faculty interviewers, Dr. Ronnie Kaye and Dr. Edward Clark, that he should have a chance in the Pharmacology Department. Years later, Craig found out that the reason he was given an interview was that they had been intrigued at the Headmaster's letter, which stated the candidate was "an unusual young man" and then repeated the statement in

capitals. On July 18 2001, Craig received the first honorary Doctor of Medicine degree from the University of Leeds for humanitarian research that has changed healthcare. The citation, presented by the Chancellor Lord Melvyn Bragg, starts: "Craig Jordan is one of the most distinguished medical scientists of the last one hundred years." He was delighted to be able to invite Drs. Clark and Kaye to the luncheon and the ceremony (Fig. 1). These were the two individuals who talent spotted Craig; Dr. Kaye was his tutor for his four years as an undergraduate, and Dr. Clark persuaded him to become a graduate student armed with the last available Medical Research Council studentship in the United Kingdom for the year 1969 (Fig. 2). Someone had declined their studentship, thus allowing Craig to do a Ph.D! Dr. Clark's project, that Craig found so attractive, was the prospect of extracting the estrogen receptor (ER) from the rodent uterus, purifying it and then crystallizing the ER protein with an estrogen and a nonsteroidal antiestrogen. The x-ray crystallography would be completed at the Astbury Department of Biophysics at the University of Leeds and all the work was estimated to take the three years of the scholarship. At that time, the nonsteroidal antiestrogens had failed to fulfill their promise in the pharmaceutical industry as "morning-after pills"; they were perfect in rats, but in women they did exactly the opposite and enhanced fertility by inducing ovulation.

The project in crystallizing the ER did not go as planned, so he rapidly changed his topic with a new title: "A study of the oestrogenic and anti-oestrogenic activities of some substituted triphenylethylenes and triphenylethanes" (Fig. 3). This was a good strategic research choice, as no one has yet succeeded in crystallizing the whole ER with either an estrogen or antiestrogen. But further difficulties were to arise in Craig's journey to a career in cancer research.

As a PhD student, Craig was talent spotted for an immediate tenure track faculty position because of his skill as a lecturer. He had no publications and his PhD topic was going nowhere. No one was recommending careers in failed contraceptives! During the interview with the University Committee charged with making the appointment, he was told that he would have to go to America to get his BTA (been to America) before he could start the job. First, however, he had to get a PhD, and to do that, it had to be exam-

ined. However, the University could find no one in the country qualified for the task. Sir Charles Dodds, the discoverer of the synthetic estrogen, diethylstilbestrol (DES), declined with regrets as he had not kept up with the literature for the past twenty years! But here is where luck and chance take control. He was in the right place at the right time and by meeting the right people, changed medicine.

Dr. Arthur Walpole was Head of the Fertility Control Program at ICI's Pharmaceuticals Division and a personal friend of the Chairman of Craig's Pharmacology Department. The University reluctantly accepted



Fig. 2. I always love dressing up! The University of Leeds is my alma mater, and I have attended four ceremonies there: (A) Bachelor of Science, First Class Honours (1969), (B) Doctor of Philosophy (1973), (C) Doctor of Science, earned by examination. A select Committee evaluated my refereed publications to establish my contribution to Science (1985) and (D) Honorary Doctor of Medicine for humanitarian research (2001).

Dr. Walpole (despite the fact that he was from industry!) to be Craig's examiner and he was also able to organize a two year visit to the Worcester Foundation for Experimental Biology (WFEB) in Shrewsbury, Massachusetts to study with Dr. Michael Harper on new methods of contraception. Harper and Walpole had completed all the early work on ICI 46,474 as a contraceptive at ICI Pharmaceuticals in the early 1960's. Craig vividly remembers the transatlantic telephone call with Dr. Harper: "Can you come in September?", "Will \$12,000 a year be enough?" and "Will you work on prostaglandins?" "Yes, yes, yes" he replied and went off to the library to find out what prostaglandins were! But when he got to the WFEB in September 1972, he was told that Dr. Harper had gone to Geneva to be Head of Contraception Research at the World Health Organization. Craig was told to sit down, write up what he would do for the next two years and organize his own laboratory. He was now an independent investigator.

A phone call to Dr. Walpole explained his dilemma at the WFEB but he felt that there was an opportunity for the failed morning-after pill, ICI 46,474 to be used for the treatment of breast cancer. This call was rewarded by Dr. Walpole arranging for funding and contacts with Ms. Lois Trench at ICI America for Craig to conduct the translational research on the drug that would become tamoxifen. As an independent Investigator, the research funding from ICI was an unrestricted research grant, but as Craig was not a cancer research scientist and he was at WFEB, the home of the oral contraceptive, what was the first step to be? Again, what's important is who you meet. After the National Cancer Act in 1971, the WFEB Director had made the decision to bring a cancer research specialist onto the Board of Scientific Advisors to help with future funding opportunities in hormones and cancer research. Dr. Elwood Jensen was the Director of the Ben May Laboratory for Cancer Research in Chicago, Illinois and was credited with the translational research where he described the ER in immature rat estrogen target tissues and then used this knowledge to propose a test for the hormone dependency of metastatic breast cancers. Simply stated, if the ER is absent in the tumor, the patient was unlikely to respond to endocrine ablation (oophorectomy, adrenalectomy or hypophysectomy), but if the tumor was ER-positive, there was a high probability that the tumor would respond to estrogen withdrawal. It was a practical test to avoid morbidity from unnecessary operations that require hospitalization.

Craig spent the day with Dr. Elwood Jensen in November 1972 and told him what he wanted to do with ICI 46,474. Craig subsequently traveled to the Ben May Laboratory for Cancer Research to be taught techniques of ER analysis and to learn all about the dimethylbenzanthracene (DMBA) rat mammary carcinoma model and then to Dr. Bill McGuire's laboratory in San Antonio, Texas to learn complementary analytical methods for the ER. Armed with these techniques and resources from ICI throughout the 1970s (his first decade of discovery), he created the laboratory principles of targeting the tumor ER and advocating the use of long term adjuvant tamoxifen therapy as the appropriate clinical strategy to save lives (Fig. 4) (Jordan and Koerner 1975; Jordan and Allen 1980).

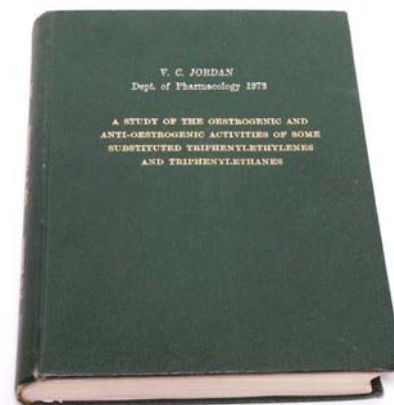


Fig. 3. My first publicity photograph during the time that I was a PhD student at the Department of Pharmacology, University of Leeds, England (1969-1972). *It was necessary as I had been selected as the Medical Research Council's student representative to the Nobel Prize Winner's Meeting in Lindau, Germany in 1972. I am examining cells from mouse vaginal smears; big science. Also shown is my PhD that nobody wanted to examine!*

This proposition by Craig was not at all popular, as throughout the 1970s and 1980s in the United Kingdom, it was strongly believed there was no correlation between tamoxifen use and the presence of the ER in breast tumors. Additionally nobody was interested in a new antihormone therapy, as combination cytotoxic chemotherapy was king. It was going to cure cancer. However, Craig persevered and had the courage of his convictions that his laboratory research would save lives. As it turned out, tamoxifen has probably saved more lives than any other cancer therapeutic drug.

Craig also learned an important lesson at the WFEB around the time he was to leave and return to Leeds. A Senior Scientist at the WFEB, Dr. Eliahu Caspi, invited Craig to his office for an interview to explore the possibility of Craig staying at the WFEB. Craig recalls this was a very frightening experience, for Dr. Caspi had a no-nonsense personality, judged people and said what he thought. He stated that he had been asked to evaluate my C.V., as everybody was of the opinion that I would be a useful asset at the WFEB. He stared at Craig across the desk and said, "You don't have a C.V., as you have no publications." After the initial shock, Craig responded, "But I haven't discovered anything yet." The advice Craig received was some of the best advice he had received thus far in his career. He was told "to tell them the story so far and link together several related publications to create a theme." Craig has done this ever since, creating the theme of tamoxifen. In 1998, with the release of the successful chemoprevention trial with tamoxifen, Craig was referred to as the "Father of Tamoxifen" by the *Chicago Tribune*, a title that has stuck to this day.

Although many people published using tamoxifen in their studies as a laboratory tool or used it in the 1960s in reproduction research, Craig's focus from the outset was clear; the goal was to develop a medicine for the treatment and prevention of breast cancer (he conducted the first chemopreventive study in the laboratory in 1974 [Jordan, 1976], three years before the drug was approved by the FDA for the treatment of metastatic breast cancer in postmenopausal women). Craig stresses that but for the unrestricted support from ICI, meeting the right people and his uncompromising



Fig. 4. The Imperial Chemical Industries (ICI) Pharmaceuticals Meeting at King's College, Cambridge in the summer of 1977. *The goal of the meeting was physician education about research being done with tamoxifen. This was the first time I presented in public my ideas about targeting the tumor estrogen receptor and using long term treatment with tamoxifen as the best strategy to be applied to adjuvant therapy (Jordan V.C., 1978. Reviews on Endocrine-related Cancer 49-55). However, the major presentation that made everything change clinically was in Arizona in 1979 (Jordan, 1979). In the above picture, Michael Baum (right), was the Chair of the session at King's College and stated that they had plans to use two years of tamoxifen as an adjuvant therapy (on a hunch). Helen Stewart (left), was considering starting a pilot trial in Scotland using five years of adjuvant tamoxifen for the treatment of patients. For the placebo arm, patients would be treated with tamoxifen at first recurrence. If toxicity was acceptable, they would move forward to test the idea of early long term treatment or late treatment at first recurrence. Both trials showed survival advantages for long term adjuvant tamoxifen. The week after the King's College Meeting, I was at the University of Wisconsin at their Comprehensive Cancer Center to convince clinicians of the Eastern Cooperative Oncology Group (ECOG) that longer was going to be better. At the time, tamoxifen was not on the market in America but I was talent spotted by Paul Carbone, the Head of ECOG and the Director of the Comprehensive Cancer Center, to be recruited to the University of Wisconsin, Department of Human Oncology. Eventually, I would be the Director of their Breast Cancer Research and Treatment Program.*

determination (many referred to this at the time as poor career judgment), tamoxifen would probably not have happened. Scientists at ICI did not conduct any studies with the drug as an antitumor agent. Indeed, in late 1972, all of the data with ICI 46,474 was reviewed and the Research Director terminated clinical trials and stopped the development project. The Marketing Department had decided that a treatment for metastatic breast cancer was not going to generate sufficient revenue.

Arthur Walpole was towards the end of his career and chose to take early retirement, but only agreed to remain an employee if funds could be given to a young man he had met, Craig Jordan, who (as he did) wanted to turn ICI 46,474 into a drug to treat breast cancer. Walpole and Craig subsequently worked together on an ICI/University joint research scheme when Craig returned as Lecturer in the Department of Pharmacology at the University of Leeds in September 1974. Earlier in his career, Dr. Walpole was an accomplished cancer research scientist, but had not been allowed to work in this area by ICI because fertility control was considered to be potentially more lucrative (Jordan, 1988). Dr. Walpole died suddenly on July 2, 1977 before he could witness the success of Craig's laboratory strategy for the treatment and prevention of breast cancer.

The clinical development of tamoxifen was very progressive and validated all your assumptions. Could you tell us how you were involved in the clinical evaluation and how you convinced the company to invest in what may have been very challenging trials?

I think it's fair to say that this was not the real story, but the real story is unbelievable. I have always considered my research as being a conversation between the laboratory and the clinic, and I had the privilege of first introducing tamoxifen to clinical trials' organizations in America. My objective was to provide a scientific rationale for the clinical studies in treatment and prevention. My research and qualifications were required to obtain approval for tamoxifen as a medicine in both Japan and Germany, and I was delighted to be the only person invited from outside of ICI Pharmaceuticals to attend a celebration in 1977, of the Queen's Award for Technological Achievement for tamoxifen. The surprising part about the tamoxifen story is that although patents for the drug were obtained by ICI Pharmaceuticals around the world, in the mid- 1960's, these same patents were denied in the United States of America. Thus, all of the work I was completing on the antitumor actions of tamoxifen in the United States was done without patent protection for ICI. Looked at another way, it was clear that all the other pharmaceutical companies had no interest in the clinical development of tamoxifen, because either the drug was not going to work very well or not generate enough revenue. But it was my clinical strategy of long term adjuvant therapy that saved lives and made revenues (Jordan, 2008 a). Clinical testing went ahead and when the patents expired in the rest of the world, ICI was awarded the patent for the use of tamoxifen in the treatment of breast cancer in 1985, but back dated to the original patent application in 1965. Now, extended adjuvant therapy was the practical solution for effective treatment. Thus, for the next twenty years, ICI was able to generate enormous revenues in the United States, as tamoxifen was the standard of care for long term adjuvant tamoxifen therapy and the only game in town. This money catalyzed the advent of ICI marketing antiandrogens for prostate cancer and the aromatase inhibitors for breast cancer.

Watching your scientific activity since the beginning, you always seem fascinated by the development of small molecules since their conception up to their development. Is that what gives you much fun in your work?

I absolutely love experiments involving the structure function relationships of the antiestrogens. My basic scientific research has been to create models of gene modulation or replication to determine the structure of the ER antiestrogen complex that subsequently could be interrogated. This passion resulted in a whole series of publications focused on the modulation of the prolactin gene (Lieberman, *et al.*, 1983 a, b; Jordan and Lieberman, 1984) which then went through a metamorphosis to study the modulation of the SERM ER complex and the way that the ligand can interact with specific amino acids, thereby switching on or switching off the complex at target genes (Wolf and Jordan, 1994). We actually found the only natural mutation of the human ER in a laboratory model of tamoxifen-stimulated tumor growth. We engineered the mutant ER into ER-negative breast cancer cells and found it would make the antiestrogen, raloxifene, an estrogen at the transforming growth factor alpha (TGF α) target gene. For me, this was important as one amino acid in the ER could change the pharmacology of raloxifene. In other words, this provided a fascinating insight into the relationship of the antiestrogenic side chain and a specific amino acid at the surface of the ER protein (Levenson and Jordan, 1998; MacGregor-Schafer, *et al.*, 2000; Liu *et al.*, 2001, 2002).

Do you think that a drug may have a commercial future in the chemoprevention of cancer?

As you know, we have made enormous progress with advancing the failed breast cancer drug, raloxifene, and millions of women are now benefiting from its use for the treatment of osteoporosis, but with a reduction in breast cancer incidence at the same time. This is the practical reality of our early translational research completed at the University of Wisconsin in the second decade of discovery (1980s). The "Tamoxifen Team" discovered selective estrogen receptor modulation and tamoxifen and raloxifene were both now classified as SERMs (Jordan, 2001). But the realization that tamoxifen could not possibly have widespread use because it increases the risk (though this is very small) of endometrial cancer in postmenopausal women (Gottardis *et al.*, 1988), naturally guided us to our new SERM strategy in the late 1980s. We discovered that SERMs maintain bone density (Jordan *et al.*, 1987) and therefore could potentially prevent osteoporosis with the beneficial antiestrogenic side effect of preventing breast cancer (Gottardis and Jordan, 1987). We had solid translational research, as we had found that tamoxifen built bone both in the laboratory (Jordan *et al.*, 1987) and in clinical trial (Love *et al.*, 1992). Raloxifene has a better safety profile and does not increase the risk of endometrial cancer (Cummings *et al.*, 1999), but it does not reduce the risk of coronary heart disease. I think the new SERM, lasofoxifene (Cummings *et al.*, 2010), is very good, as it prevents osteoporosis, breast cancer, coronary heart disease and strokes, but without an increase of endometrial cancer. The problem is how to advance in a crowded market with low budgets for marketing. Lasofoxifene is approved but not marketed in the European Union.

No molecule targeting estrogen receptor has, to date, proved to be more efficient than tamoxifen in patients despite the development of a number of promising compounds. How do you

explain that? Was it a choice of the pharmaceutical industry because of the cost of the development of such a compound?

The issue with tamoxifen is unique. It was clearly lucky that tamoxifen had an acceptable toxicology profile for the treatment of cancer. It came onto the market at a time when the standard of care was combination cytotoxic chemotherapy, so tamoxifen looked good to patients. Tamoxifen was not supposed to succeed, but advanced from strength to strength for twenty years. However, things change very rapidly in the arena of patient preference. In the early 1990s, when tamoxifen was being considered for testing as a chemopreventive and the specter of endometrial cancer translated from the laboratory (Gottardis *et al.*, 1988) to clinical practice, this was clearly not good news for well women. Worse still, tamoxifen was found to produce DNA adducts in rat liver and initiate rat liver hepatocarcinogenesis (Jordan, 1995). Although liver tumors did not translate to clinical practice, this did not lessen concern, as the drug ended up with a black box label as a human carcinogen. Timing is everything with discovery and competitors could never catch up with clinical testing, despite the fact they may have been safer. We will never know.

To demonstrate that natural or synthetic molecules can prevent the occurrence of cancer is long and expensive. This raises the question of the life of the patents but also the natural molecules, which may not be patentable. Do you think there may be solutions to these problems?

I think it's currently impossible to find a solution to this dilemma. Clearly, the pharmaceutical industry will never advance with twenty year studies because the patents will run out. But here is a controversial point: the success of health care has now created the situation of increased longevity, so that drugs that enhance survival through prevention can only make matters worse. What is society to do? How does society find the resources to support an aging population?

You have developed recently a very provocative approach using estrogens for the treatment of breast cancers. This can be considered as a paradoxical use of estrogens? Could you explain to us a little bit about that.

The third and fourth decades have been a wonderful surprise in our journey of discovery. We posed the question (based upon the clinical acceptance of long term antihormonal therapy (Jordan, 2008 a) as the most appropriate adjuvant treatment for breast cancer): what is the mechanism and the timeframe for acquired antihormone resistance? Our first model clearly showed something unique as far as drug resistance is concerned—SERM-stimulated growth, something that is not seen with any other drug in cancer therapy (Gottardis and Jordan, 1988). This form of resistance occurred within a year or two and was consistent with the development of acquired resistance to tamoxifen in metastatic breast cancer. However, here was the dilemma: this model did not replicate the outstanding success observed with five years of adjuvant tamoxifen treatment (Early Breast Cancer Trialists' Collaborative Group (EBCTG), 2011). In fact, five years of treatment continues to enhance decreases in mortality for more than a decade once tamoxifen is stopped. By a series of lucky accidents, one of my students (Doug Wolf) discovered that physiologic estrogen could cause dramatic tumor regression after five years of tamoxifen treatment, i.e. serial transplantation of tamoxifen-resistant tumors into generations of

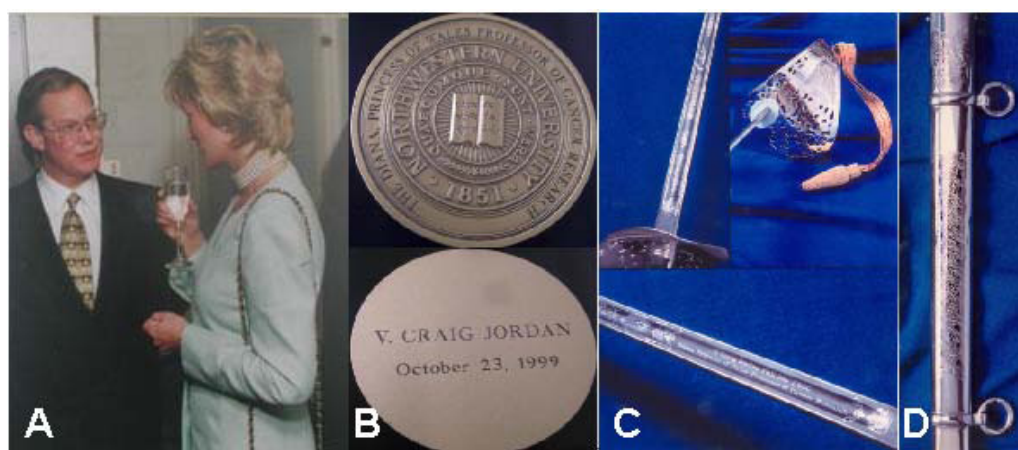


Fig. 5. The Diana, Princess of Wales Chair of Cancer Research.

In June 1996, Diana, the Princess of Wales visited Chicago for three days and we first met (A) at the evening reception at the home of the President of Northwestern University, Henry Bienen. The Chair was anonymously endowed at the Robert H. Lurie Comprehensive Cancer Center after Diana's untimely death on August 31, 1997. I was inaugurated on October 23, 1999, being presented with a unique Professorial medal (B) with copies being sent to her sons Prince William and Harry and also kept by my daughters, Helen and Alexandra. My students presented me with an

engraved sword (C) to commemorate the event and their names and the dates of the award of their PhD degrees are engraved on the scabbard (D).

tamoxifen-treated mice (Wolf and Jordan, 1993). This discovery reminded me of the words of Sir Alexander Haddow, FRS in 1970 during the Inaugural Karnofsky Lecture at the American Society of Clinical Oncology (ASCO): "...the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us..." (Haddow, 1970). It is now clear that aggressive estrogen deprivation with aromatase inhibitors or SERMs can rapidly re-configure breast cancer cells through an evolution of drug resistance, which exposes a vulnerability that could not be anticipated—physiological estrogen induced apoptosis (Yao, 2000; Lewis *et al.*, 2005). When Haddow did his original work using high dose DES for the treatment of metastatic breast cancer in women during their late sixties and seventies, the best therapeutic results occurred the further away the patient was from the menopause. Antihormone therapy accelerates all of that in breast cancer, so physiologic estrogen can initiate the same triggering mechanism. Indeed, this is possibly the same mechanism that is occurring in the Women's Health Initiative (WHI) by conjugated equine estrogen (CEE) alone actually produces a decrease in the incidence of breast cancer in hysterectomized postmenopausal women (La Croix *et al.*, 2011). What is particularly interesting about these data is the six years of monitoring after CEE is stopped, there is a continued reduction in the incidence of breast cancer, i.e. the estrogen has destroyed the nascent breast cancer cells in the ducts (Jordan and Ford, 2011). Our current laboratory work is focused entirely on deciphering the molecular mechanism of estrogen-induced apoptosis (Ariazi, *in press*). In this way, we may find the vulnerability triggered by the ER estrogen complex for cellular destruction; that vulnerable site in the cancer cell may be the next target for a new class of selective anticancer agents applicable to sites other than breast cancer.

Your contributions to medicine have received a lot of recognition (Table 1) but how does one become the "Diana, Princess of Wales Professor of Cancer Research"?!

Life is all about chance meetings. In the mid-1990s, I was invited to organize a Breast Cancer Symposium in Chicago, and Diana was my Keynote Speaker (Fig. 5). She came on a three day visit to Northwestern University and the Robert H. Lurie Comprehensive

Cancer Center. Naturally, it was a very special time and when she left to return to London, we agreed to correspond and I sent her copies of my books on tamoxifen. There was even talk of a return trip for either her or Prince William or Prince Harry, to open one of our new research buildings. Regrettably, everything changed with her untimely death in a tragic car accident in Paris on August 31, 1997. An anonymous donation was subsequently made to the Robert H. Lurie Comprehensive Cancer Center, and with letters from Lady Sarah McCorquodale, (her sister) and the Earl Spencer (her brother), it was agreed that I would hold a Professorship at

TABLE 1

AWARDS & HONORS

St. Gallen International Breast Cancer Prize	2011
Elected to the National Academy of Sciences, USA (Fig. 6)	2009
Elected Fellow of the Academy of Medical Sciences (UK equivalent of Inst. of Medicine in the US)	2009
Elected Fellow of the Society of Biology (UK)	2009
Honorary Doctor of Medicine Degree, University of Crete, Greece	2009
39 th David A. Karnofsky Award, ASCO	2008
Honorary Fellowship of the Royal Society of Medicine (Fig. 7)	2008
Honorary Member of the Royal Pharmaceutical Society of Great Britain	2008
Gregory Pincus Award and Medal, Worcester Foundation for Biomedical Research, U. Mass	2007
American Cancer Society Award for Chemoprevention, ASCO	2006
Honorary Doctor of Science Degree, University of Bradford, England	2005
Alfred G. Knudson Jr. Chair in Basic Science, Fox Chase Cancer Center	2004
3 rd George and Christine Sosnovsky Award in Cancer Therapy, Royal Society of Chemistry	2003
The Kettering Prize, General Motors Cancer Research Foundation	2003
Officer of the Most Excellent Order of the British Empire (OBE) Services to International Breast Cancer Research	2002
American Cancer Society Medal of Honor	2002
Inaugural Dorothy P. Landon AACR Prize in Translational Research	2002
Bristol Myers Squibb Award for Distinguished Achievement in Cancer Research	2001
Honorary Doctor of Medicine Degree, University of Leeds	2001
European Institute of Oncology Breast Cancer Therapy Award	2001
Honorary Doctor of Science Degree, University of Massachusetts	2001
Honorary Faculty Fellowship Award, University College, Dublin	2000
Diana, Princess of Wales Professor of Cancer Research, Robert H. Lurie Comprehensive Cancer Center	1999

Northwestern University in her name. Essentially, it was my British citizenship, a British medicine (tamoxifen), and our meeting and correspondence that was important to the family. On October 23, 1999, the Professorship was conferred on me by Henry Bienen, the President of Northwestern University and over a two day period, there was a Symposium in my honor by my former PhD students and during the celebration dinner, attended by representatives from the British Embassy, Barry Furr (the Chief Scientist from ICI), family, friends and colleagues, my students presented me with an engraved sword (Fig. 5) with each of the dates of their Ph.D engraved on the scabbard as battle honors—very moving!

You have contributed more than 600 research and review papers to the literature with more than 23,000 citations and an h-index of 80. If you had to select ten of your research papers and three reviews, which would they be and why?

Jordan V.C. (1976). *Eur J Cancer* 12: 419-424. Literally my first cancer research paper with tamoxifen that was rejected in 1974, but with kind and generous comments from one of the reviewers. I persevered and eventually this was one of the papers from my work used to justify the chemoprevention trials.

Jordan V.C. and Allen K.E. (1980). *Eur J Cancer* 16: 239-251. The paper makes three points: 1. this is the first refereed article that longer treatment is going to be better than shorter treatment; 2. our discovery of 4-hydroxytamoxifen's pharmacology indicating it to be a potent antiestrogen with a binding affinity for ER equivalent to estradiols (Jordan *et al.*, 1977), naturally made us think that this would be a more powerful anticancer agent—not true, it cleared too quickly and 3. finally, we stated that antiestrogen treatment followed by estrogen deprivation would be a good strategy for people—true.

Gottardis M.M., *et al.*, (1988). *Cancer Res* 48: 812-815. This was the paper that warned the clinical community that tamoxifen could potentially increase the incidence of endometrial cancer in patients—true.

Gottardis M.M. and Jordan V.C. (1988). *Cancer Res* 48: 5183-5187. This was the first report that acquired drug resistance with tamoxifen was unique and stimulated by SERMs—true.

Love R.R., *et al.*, (1992). *New Engl J Med* 326: 852-856. This was the randomized clinical trial based on our laboratory evidence and subsequently those of others that tamoxifen would maintain bone density in people. This paper opened the door to raloxifene.

Levenson A.S. and Jordan V.C. (1998). *Cancer Res* 58: 1872-1875. A clean demonstration that a mutant ER found in a tamoxifen-stimulated tumor by a previous PhD student (Doug Wolf) could change an antiestrogen to an estrogen. This could be done by a natural process.

Cummings S.R., *et al.*, (1999). *JAMA* 281: 2189-2197. Proof of principle that the concept we first

articulated back in the late 1980s that you could develop a SERM to prevent osteoporosis and prevent breast cancer at the same time—true.

Yao K., *et al.*, (2000). *Clin Cancer Res* 6: 2028-2036. The first refereed publication to demonstrate that drug resistance to tamoxifen evolves and exposes a vulnerability to permit physiologic estrogen to cause tumor regression. Subsequently translated to the clinic—true.

Vogel V.G., *et al.*, (2006). The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA*. 295: 2727-2741. Two discarded drugs from the pharmaceutical industry that were re-invented in the same pharmacology laboratory to become the pioneering chemopreventive agents and FDA-approved—true.

Vogel V.G., *et al.*, (2010). *Cancer Prev Res* 3: 696-706. A follow-up of the trial several years after stopping SERM treatment, confirmed the predictions of one of my PhD students (Marco Gottardis) in 1987 that tamoxifen would be the better chemopreventive in the long term.

I've always viewed an invitation to write a review article from a journal as a wonderful opportunity to project your personality, express your views and most importantly, reach out to young scientists and graduate students as theirs is the future. Here are my three choices:

Jordan V.C. (1984). *Pharm Rev* 36: 245-276. This was my first major review when I first came to America. No one had really treated the topic as an issue in pharmacology, as all of the previous reviews in the 1960s and 1970s were about the control of fertility. I wanted a summary of the mechanisms of action of antiestrogens. It was all of our knowledge up to that point (423 citations).

Jordan V.C. (2006). *Br J Pharmacol* 147: S269-S276. I was thrilled to be asked by the British Pharmacological Society to write the story of my research in a Special Issue of our Journal. I got

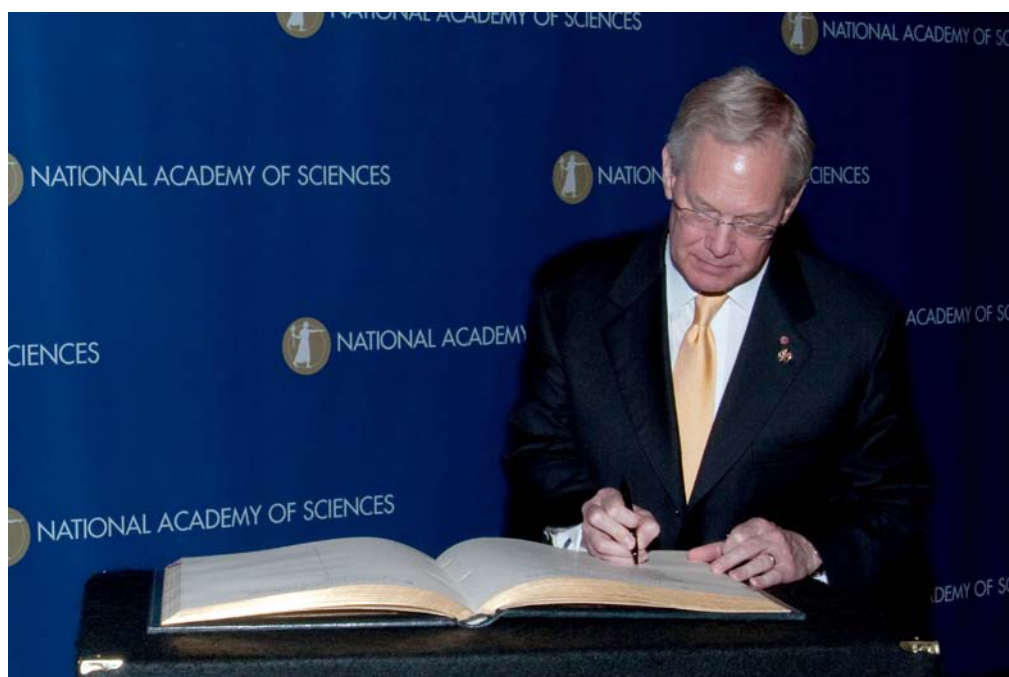


Fig. 6. Signing the "Great Book" of Members of the National Academy of the Sciences USA during the Induction Ceremony on April 24, 2010.

wonderful feedback from students.

Jordan V.C. (2009). *Cancer Res.* 69: 1243-1254. I was proud to be asked by the American Association for Cancer Research (AACR) to contribute a review of progress in hormone dependent tumors as a part of a series to celebrate the 100th anniversary of AACR.

I see that you received the David A. Karnofsky Award in 2008 from ASCO, but it is stated in the regulations for the Award that it is given in “recognition of innovative clinical research and developments that have changed the way oncologists think about the general practice of oncology.” You are a laboratory scientist and not a clinician; didn’t this surprise you?

When I received the telephone call from the Chair of the Awards Committee, Gabriel Hortobagyi, I was absolutely dumbfounded, because naturally, I knew I was not a clinician! All previous recipients were clinicians. This is ASCO’s highest award, and I was being asked to join the legends of clinical practice. For the first fifteen minutes of my conversation with Gabriel, I examined with him every reason why I should not be their recipient. After fifteen minutes, he became exasperated and said, “Is this a yes, I accept?” I accepted the honor. Apparently, I learned, the reason the Committee selected my work was because as a laboratory scientist and a pharmacologist, I had always been present at clinical breast

cancer meetings over the decades, putting forward my point-of-view in cancer treatment with SERMs. For me, the promise of life was the most important goal. But safety was essential. The involvement I had every day with the clinical evaluation of tamoxifen (Love *et al.*, 1992), followed by leadership positions for the evaluation of raloxifene (Cummings *et al.*, 1999), and then as the Scientific Chair of the Study of Tamoxifen and Raloxifene (STAR) (Vogel *et al.*, 2006, 2010) allowed me to deploy the knowledge generated by my “Tamoxifen Team” over decades to save lives and advance women’s health (Jordan, 2008 b). Please remember that when I started this improbable and unlikely journey at the beginning of the 1970s, cancer therapeutics with a targeted agent, chemoprevention, and the drug group, SERMs (or even tamoxifen for that matter!) did not exist. Cancer research was not recommended as a career for the pharmacologist and the pharmacologist would not knowingly venture into women’s health. All of the revenues in the pharmaceutical industry were derived from heart drugs and drugs that affected the central nervous system (e.g. tranquilizers, etc.).

When I was starting the research for my PhD at Leeds University, Sir Alexander Haddow, FRS in the Inaugural Karnofsky Lecture (Haddow, 1970), was dismayed at the prospect for cancer therapeutics. Unlike the success noted with antibiotics for the treatment of different infectious diseases, there were no laboratory tests to

establish whether a chemotherapy would be effective or not. The physician just had to give it to the patient and see if it worked! Haddow was also not convinced that a cancer-specific drug could be developed because cancer was self. In Haddow’s Karnofsky Lecture publication, there was one glimmer of hope: Haddow had used the first chemical therapy to treat any cancer, i.e. high dose estrogen to treat metastatic breast cancer in women in their late sixties and seventies. He observed that some of the responses just melted the tumors away. But he was dismayed that the mechanisms had remained elusive. I am pleased to say that we have now solved the question surrounding the mechanism of estrogen-induced apoptosis (Ariazi, in press).

It is fair to say that the work that has evolved and developed on the treatment and prevention of breast cancer over the past four decades has changed our outlook and replaced pessimism with hope. The first decade of discovery was essential to move forward in the field (Jordan, 2008 a). It has not only been possible to create change in medical practice, but the laboratory principles all translated to patient care to save or at least extend lives. That is what pharmacology is.

In closing, I must end where we began. I have thanked Drs. Kaye and Clark (Fig. 1) many times for the opportunity they gave me with a place at Leeds University. The reply I received was usually “we were only doing our job.” Good words to remember and live by.



Fig. 7. Honorary Fellowship of the Royal Society of Medicine awarded by Professor Ilora Finlay, Baroness Finlay of Llandaff, President of the Royal Society of Medicine (2008). This honor is awarded to individuals of international standing who have eminently distinguished themselves in the service of medicine and the fields which influence it. The Society permits, at most, 100 people into this elite group at any one time. In 2008 there were only 89 Honorary Fellows worldwide. In 2009, I received the Jephcott Medal from the Royal Society of Medicine, and in 2010, I was elected as the President of the Royal Society of Medicine Foundation in North America.

Acknowledgements

Dr. Jordan wishes to thank all of his “Tamoxifen Teams”, who for the past four decades have converted ideas into lives saved. Dr. Jordan wishes to thank the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen For The Cure Foundation under Award number SAC100009

and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008 for his current research funding. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

References

- ARIAZI E.A., CUNLIFFE H.E., LEWIS-WAMBI J.S., SLIFKER M.J., WILLIS A.L., RAMOS P., TAPIA C., KIM H.R., YERRUM S., SHARMA C.G.N., NICOLAS E., BALAGURUNATHAN Y., ROSS E.A. and JORDAN V.C. (in press). Estrogen Induces Apoptosis in Estrogen Deprivation-resistant Breast Cancer via Stress Responses as Identified by Global Gene Expression. *Proc Natl Acad Sci USA*.
- CUMMINGS S.R., ECKERT S., KRUEGER K.A., GRADY D., POWLES T.J., CAULEY J.A., NORTON L., NICKELSEN T., BJARNASON N.H., MORROW M., LIPPMAN M.E., BLACK D., GLUSMAN J.E., COSTAA. and JORDAN V.C. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. *JAMA* 281: 2189-2197.
- CUMMINGS S.R., ENSRUD K., DELMAS P.D., REID D.M., GOLDSTEIN S., SRIRAM U., LEE A., THOMPSON J., ARMSTRONG R.A., THOMPSON D.D., POWLES T., ZANCHETTA J., KENDLER D., NEVEN P. and EASTELL R. (2010). Lasofoxifene in postmenopausal women with osteoporosis. *New Engl J Med* 362: 686-696.
- EARLY BREAST CANCER TRIALISTS' COLLABORATIVE GROUP (EBCTCG). (2011). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 378: 771-784.
- GOTTARDIS M.M. and JORDAN V.C. (1987). The antitumor actions of keoxifene (raloxifene) and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47: 4020-4024.
- GOTTARDIS M.M. and JORDAN V.C. (1988). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48: 5183-5187.
- GOTTARDIS M.M., ROBINSON S.P., SATYASWAROOP P.G. and JORDAN V.C. (1988). Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48: 812-815.
- HADDOW A. (1970). David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26: 737-754.
- JORDAN V.C. and KOERNER S. (1975). Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 11: 205-206.
- JORDAN V.C. (1976). Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer* 12: 419-424.
- JORDAN V.C., COLLINS M.M., ROWSBY L. and PRESTWICH G. (1977). A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* 75: 305-316.
- JORDAN V.C. (1978). Use of the DMBA-induced rat mammary carcinoma system for the evaluation of tamoxifen as a potential adjuvant therapy. *Rev on Endocr Relat Cancer* 49-55.
- JORDAN V.C., DIX C.J. and ALLEN K.E. (1979). The effectiveness of long term tamoxifen treatment in a laboratory model for adjuvant hormone therapy of breast cancer. In *Adjuvant Therapy of Cancer II* (Eds. S.E. Salmon and S. E. Jones). Grune & Stratton Inc., Philadelphia, pp. 19-26.
- JORDAN V.C. and ALLEN K.E. (1980). Evaluation of the antitumour activity of the nonsteroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 16: 239-251.
- JORDAN V.C. and LIEBERMAN M.E. (1984). Estrogen-stimulated prolactin synthesis *in vitro* classification of agonists, partial agonist and antagonist actions based on structure. *Mol Pharm* 26: 279-285.
- JORDAN V.C. (1984). Biochemical pharmacology of antiestrogen action. *Pharm Rev* 36: 245-276.
- JORDAN V.C., PHELPS E. and LINDGREN J.U. (1987). Effects of antiestrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10: 31-35.
- JORDAN V.C. (1988). The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L. Walpole. *Breast Cancer Res Treat* 11: 197-209.
- JORDAN V.C. (1995). What if tamoxifen (ICI 46,474) had been found to produce liver tumors in rats in 1973? *Ann Oncol* 6: 29-43.
- JORDAN V.C. (2001). Selective Estrogen Receptor Modulation: a personal perspective. (Perspectives in Cancer Research). *Cancer Res* 61: 5683-5687.
- JORDAN V.C. (2003). Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2: 205-213.
- JORDAN V.C. (2006). 75th Anniversary Edition British Journal of Pharmacology Special Issue. Tamoxifen (ICI 46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 147: S269-S276.
- JORDAN V.C. (2007). Chemoprevention of Breast Cancer with Selective Oestrogen Receptor Modulators. *Nat Rev Cancer* 7: 46-53.
- JORDAN V.C. (2008 a). Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 44: 30-38.
- JORDAN V.C. (2008 b). The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer—survival or death? *J Clin Oncol* 26: 3073-3082.
- JORDAN V.C. (2009). A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 69: 1243-1254.
- JORDAN V.C. and FORD L.S. (2011). Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-Induced Apoptosis. *Cancer Prev Res* 4: 633-637.
- LACROIX A.Z., CHLEBOWSKI R.T., MANSON J.E., ARAGAKIA K., JOHNSON K.C., MARTIN L., MARGOLIS K.L., STEFANICK M.L., BRZYSKI R., CURB J.D., HOWARD B.V., LEWIS C.E. and WACTAWSKI-WENDE, J. (2011). Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 205: 1305-1314.
- LEVENSON A.S. and JORDAN V.C. (1998). The key to the antiestrogenic mechanism of raloxifene is Amino Acid 351 (Asp) in the estrogen receptor. *Cancer Res* 58: 1872-1875.
- LEWIS J.S., MEEKE K., OSIPO C., ROSS E.A., KIDAWI N., LI Y., BELL E., CHANDEL N.S. and JORDAN V.C. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97: 1746-1759.
- LIEBERMAN M.E., JORDAN V.C., FRITSCH M., SANTOS M.A. and GORSKI J. (1983 a). Direct and reversible inhibition of estradiol-stimulated prolactin synthesis by antiestrogens *in vitro*. *J Biol Chem* 258: 4734-4740.
- LIEBERMAN M.E., GORSKI J. and JORDAN V.C. (1983 b). An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens *in vitro*. *J Biol Chem* 258: 4741-4745.
- LIU H., LEE E.S., DE LOS REYESA., ZAPF J.W. and JORDAN V.C. (2001). Silencing and reactivation of the selective estrogen receptor modulator (SERM)-ER alpha complex. *Cancer Res* 61: 3632-3639.
- LIU H., PARK W., BENTREM D.J., MCKIAN K.P., DE LOS REYESA., MACGREGOR-SCHAFER J., ZAPF J. and JORDAN V.C. (2002). Structure-function relationships of the raloxifene-estrogen receptor alpha complex for regulating transforming growth factor alpha expression in breast cancer cells. *J Biol Chem* 277: 9189-9198.
- LOVE R.R., MAZESS R.B., BARDEN H.S., EPSTEIN S., NEWCOMB P.A., JORDAN V.C., CARBONE P.P. and DEMETS D.L. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *New Engl J Med* 326: 852-856.
- MACGREGOR-SCHAFER J.I., LIU H., BENTREM D., ZAPF J. and JORDAN V.C. (2000). Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex by substituting glycine for aspartate at amino acid 351. *Cancer Res* 60: 5097-5105.
- VOGEL V.G., COSTANTINO J.P., WICKERHAM D.L., CRONIN W.M., CECCHINI R.S., ATKINS J.N., BEVERS T.B., FEHRENBACHER L., PAJON E.R., WADE J.L., ROBIDOUX A., MARGOLESE R.G., JAMES J., LIPPMAN S.M., RUNOWICZ C.D., GANZ P.A., REIS S.E., MCCASKILL-STEVENS W., FORD L.G., JORDAN V.C. and WOLMARK N. (2006). The Study of Tamoxifen and Raloxifene (STAR): Report of the National Surgical Adjuvant Breast and Bowel Project P-2 Trial. *JAMA* 295: 2727-2741.
- VOGEL V.G., COSTANTINO J.P., WICKERHAM D.L., CRONIN W.M., CECCHINI R.S., ATKINS J.N., BEVERS T.B., FEHRENBACHER L., PAJON E.R., WADE J.L., ROBIDOUX A., MARGOLESE R.G., JAMES J., RUNOWICZ C.D., GANZ P.A., REIS S.E., MCCASKILL-STEVENS W., FORD L.G., JORDAN V.C. and WOLMARK N. (2010). Update of the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing Breast Cancer. *Cancer Prev Res* 3: 696-706.

WOLF D.M. and JORDAN V.C. (1993). A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 127: 23-33.

WOLF D.M. and JORDAN V.C. (1994). The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding

domain. *Breast Cancer Res Treat* 31: 129-138.

YAO K., LEE E.S., BENTREM D.J., ENGLAND G., SCHAFER J.I.M., O'REGAN R. and JORDAN V.C. (2000). Antitumor action of physiologic estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6: 2028-2036.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Estrogenic in vitro assay on mouse embryonic Leydig cells.

Gina La Sala, Donatella Farini and Massimo De Felici.
Int. J. Dev. Biol. (2010) 54: 717-722

Estrogen regulation of placental angiogenesis and fetal ovarian development during primate pregnancy.

Eugene D. Albrecht and Gerald J. Pepe.
Int. J. Dev. Biol. (2010) 54: 397-407

Casein kinase I epsilon somatic mutations found in breast cancer cause overgrowth in *Drosophila*.

Tomas Dolezal, Katerina Kucerova, Jana Neuhold and Peter J. Bryant.
Int. J. Dev. Biol. (2010) 54: 1419-1424

Epithelial-Mesenchymal Transitions in development and disease: old views and new perspectives

M. Angela Nieto.
Int. J. Dev. Biol. (2009) 53: 1541-1547

The expression of the imprinted gene *Ipl* is restricted to extra-embryonic tissues and embryonic lateral mesoderm during early mouse development.

Sally L Dunwoodie and Rosa S P Beddington
Int. J. Dev. Biol. (2002) 46: 459-466

Germ cells, gonads and sex reversal in marsupials.

M B Renfree and G Shaw.
Int. J. Dev. Biol. (2001) 45: 557-567

Detection of sex-specific proteins in chick embryo gonads and mesonephros: effects of estradiol benzoate or tamoxifen on their expression.

R Didier and Y Croisille.
Int. J. Dev. Biol. (1989) 33: 467-475

5 yr ISI Impact Factor (2010) = 2.961





Review

Progress in endocrine approaches to the treatment and prevention of breast cancer

Ifeyinwa Obiorah¹, V. Craig Jordan*

Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Rd NW, Research Building, Suite E208, Washington, DC 20057, United States

ARTICLE INFO

Article history:

Received 31 August 2011
Received in revised form 8 September 2011
Accepted 10 September 2011

Keywords:

Selective estrogen receptor modulator
Tamoxifen
Aromatase inhibitors
Estrogen receptor

ABSTRACT

Tamoxifen had been the only available hormonal option for the systemic treatment for breast cancer from 1973 to 2000. Enormous efforts have led to the development of potent and selective third generation aromatase inhibitors including anastrozole, letrozole and exemestane. Due to their superior efficacy to tamoxifen, aromatase inhibitors are presently approved as first line agents for the treatment of advanced estrogen receptor (ER) positive breast cancer and adjuvant therapy in early ER positive early breast cancer in postmenopausal women. Selective ER Modulators (SERMS), tamoxifen and raloxifene are the only agents presently used in breast cancer prevention in high risk women and their use has increased substantially over the last decade. Third generations SERMS, lasofoxifene and bazedoxifene have shown significant reduction in bone loss compared to placebo in postmenopausal women and are currently approved in the European Union for the treatment of postmenopausal osteoporosis. This review outlines the current strategies employed in the use of endocrine therapy in the management and prevention of breast cancer.

© 2011 Elsevier Ireland Ltd. All rights reserved.

Contents

1. Introduction.....	315
2. Tamoxifen versus aromatase inhibitors.....	316
2.1. Advanced breast cancer.....	316
2.2. Adjuvant monotherapy.....	316
2.3. Sequential therapy.....	316
2.4. Extended therapy.....	318
3. The SERM concept and breast cancer prevention.....	318
4. New generation SERMS.....	318
5. Conclusion.....	319
Contributors.....	320
Competing interest and funding information.....	320
Provenance and peer review.....	320
Acknowledgments.....	320
References.....	320

* Corresponding author at: Comprehensive Cancer Center, Department of Oncology, Georgetown University Medical Center, 3970 Reservoir Rd NW, Research Building, Suite E501, Washington, DC 20057, United States. Tel.: +1 202 687 2897; fax: +1 202 687 6402.

E-mail addresses: oi3@georgetown.edu (I. Obiorah), vcj2@georgetown.edu (V.C. Jordan).

¹ Tel.: +1 202 687 4842.

1. Introduction

The journey to determine the mechanism that lies behind the growth of breast cancer started more than 100 years ago. The first medical evidence was the suppression of estrogen levels through oophorectomy to cause regression of metastatic breast cancer [1].

Similar antitumor effects were observed following adrenalectomy and hypophysectomy in postmenopausal women with breast cancer [2]. This led to the evolution of endocrine therapies, with the principal goal of depriving tumor cells of estrogen to induce tumor regression. The story of the reinvention of tamoxifen to become the gold standard for the adjuvant treatment of breast cancer and the pioneering medicine for the reduction of breast cancer incidence in high risk women, has been told in detail elsewhere [3,4]. The translational laboratory research work in the 1970s [5] catalyzed the move from orphan drug for the adjuvant treatment and prevention of breast cancer resulting in tamoxifen becoming the standard of care for the long term adjuvant therapy of ER positive breast cancer and the extension of the lives of millions of women worldwide. Despite the clinical success of tamoxifen, development of drug resistance and endometrial cancer led to the requirement of alternative hormonal therapy to avoid these issues. The clinical efficacy of third generation non steroidal aromatase inhibitors (AIs), anastrozole and letrozole and steroidal AI, exemestane has been extensively studied in comparison to tamoxifen. Although AIs have shown some superiority to tamoxifen as first-line agents in the treatment of postmenopausal women with breast cancer [6–8] selective estrogen receptor modulators (SERMs) remain the mainstay of treatment in breast cancer prevention. In this review, we focus on current published data on the treatment strategies using hormonal therapy in the treatment and prevention of breast cancer.

2. Tamoxifen versus aromatase inhibitors

2.1. Advanced breast cancer

A meta-analysis [9] of comparative studies of AIs with tamoxifen, in postmenopausal women with advanced breast cancer demonstrated a significant difference favoring AIs over tamoxifen as first line agents in overall response rate (ORR; OR, 1.56; 95% CI, 1.17–2.07; $p=0.002$) and clinical benefit (CB; OR, 1.70; 95% CI, 1.24–2.33; $p=0.0009$). Although the overall survival (OS) was increased for the AI arm compared to the tamoxifen arm, the differences observed were not statistically significant (OR, 1.95; 95% CI, 0.88–4.30; $p=0.10$).

2.2. Adjuvant monotherapy

In estrogen receptor (ER) positive early breast cancer, 5 years of adjuvant tamoxifen significantly reduces breast cancer recurrence and mortality throughout the first 10 years and 15 years respectively [10]. Incorporation of AIs as adjuvant therapy in breast cancer has been extensively studied. Several randomized trials [11–13] have compared AIs to 5 years of tamoxifen as primary adjuvant treatment of postmenopausal women with early breast cancer. The results are summarized in Table 1. Although anastrozole and letrozole showed significant improvements for disease free survival (DFS) and time to distant recurrence (TTDR) and exemestane only improved TTDR, none of the AIs showed significant overall survival (OS). A meta-analysis of the ATAC and BIG trials [14] revealed that the AIs achieved a 2.9% absolute decrease in recurrence (9.6% for AI vs. 12.6% for tamoxifen; $p<0.00001$) and a nonsignificant reduction in breast cancer mortality. In both studies, the incidence of bone fractures was observed more frequently in the AI arm but gynecological problems were more frequent with tamoxifen therapy. At 10 year follow up of the ATAC trial, the incidence of most cancers was similar between groups and continue to be increased with anastrozole for colorectal (66 vs. 44; OR 1.51, 1.01–2.27) and lung cancer (51 vs. 34; OR 1.51, 95% CI 0.96–2.41), and lower for endometrial cancer (6 vs. 24; OR 0.25, 95% CI 0.08–0.63), melanoma (8 vs. 19; 0.42, 0.16–1.00), and ovarian cancer (17 vs. 28). Although

long term effects of AIs are not yet established, it is suggested that bisphosphonates be added to AIs regimens to prevent AI associated bone loss. Furthermore, concerns have been raised about the potential increase of myocardial infarction with AIs. This has been addressed in clinical trials, which revealed no significant difference between AIs and tamoxifen [15]. However combined analysis [16] of multiple randomized controlled trials comparing AIs to tamoxifen, demonstrated that AIs were associated with a higher incidence of grade 3 and 4 cardiovascular events ($p=0.038$) while thromboembolic events were more frequent in the tamoxifen arm ($p<0.0001$).

2.3. Sequential therapy

It is well known that despite an initial response to tamoxifen, disease progression can occur due to acquired resistance. Prevention of breast cancer recurrences and improvement of survival have been explored with the use of sequential therapy with AIs after 2–3 years of tamoxifen to a total of 5 years of endocrine therapy. Pooled analysis [14] of 4 trials [15,17,18] in which 2–3 years of tamoxifen is switched to either 2–3 years of AIs or tamoxifen revealed that AI therapy was associated with an absolute 3.1% (SE = 0.6%) reduction in recurrence (5% for AI vs. 8.1% for tamoxifen; $2p<0.00001$) and an absolute 0.7% (SE = 0.3%) decrease in breast cancer mortality (1.7% for AI vs. 2.4% for tamoxifen; $2p=0.02$) after approximately 5 years of hormonal therapy. Whereas breast cancer mortality was significantly reduced, none of the individual trials reported a significant overall survival. However, updated data from the Anastrozole-Nolvadex (ARNO)-95trial, showed significant reduction in the risk of recurrences ($p=0.049$) and improved overall survival ($p=0.045$) with sequential treatment with anastrozole compared to tamoxifen monotherapy [19].

Two studies compared primary AI monotherapy with sequential therapy including tamoxifen followed by an AI. In addition to assessment of letrozole monotherapy compared to tamoxifen, the BIG 1-98 trial [12] also evaluated sequential therapy of 2 years of letrozole followed by 3 years of tamoxifen or 2 years of tamoxifen followed by 3 years of letrozole. A median follow up of 71 months revealed that there was no significant difference in terms of DFS with either sequential therapy when compared with letrozole alone. The TEAM trial was initially designed to evaluate the clinical efficacy of exemestane compared to 5 years of tamoxifen as initial adjuvant endocrine therapy. The study design was changed, based on the results of the Intergroup Exemestane Study (IES) trial, to include the sequential use of exemestane after 2.5–3 years of tamoxifen treatment. Updated analysis from the TEAM trial [20] at 5.1 years follow up showed that there was no significant difference in DFS between exemestane alone and tamoxifen followed by exemestane (Fig. 1).

Therefore current recommendation in adjuvant endocrine treatment of ER positive breast cancer (Fig. 2.) is that postmenopausal women take AIs as a primary agent for 5 years or for 2–3 years after tamoxifen, while tamoxifen is recommended as a first line treatment for pre or peri-menopausal women [21]. However which AI to use as either initial or sequential adjuvant therapy is yet to be determined. Studies [22] have shown that letrozole was more potent than anastrozole in the inhibition of aromatization and estrogen suppression in postmenopausal women with locally advanced and invasive ER positive breast cancer. But the superiority of letrozole was not observed in the head to head comparison of letrozole and anastrozole as second line agents in metastatic breast cancer [23]. The ACSOG trial [24] compared the clinical efficacy of all three AIs in the neoadjuvant treatment of locally advanced breast cancer. Preliminary results showed no significant difference in the clinical

Table 1

Third generation aromatase inhibitors versus tamoxifen as first line adjuvant therapy.

TRIAL	ARM	Median follow-up (months)	n	DFS	TTDR
ATAC [11]	ANA vs.TAM	120	6241	HR 0.91, 95% CI 0.83–0.99 $p=0.04$	HR 0.87, 95% CI 0.77–0.99 $p=0.03$
BIG [12]	LET vs. TAM	76	4922	HR 0.88, 95%CI 0.78–0.99 $p=0.03$	HR 0.85, 95% CI 0.72–1.00 $p=0.05$
TEAM [13]	EXE vs. TAM	33	9766	HR 0.91, 95% CI 0.83–0.99 $p=0.12$	HR 0.81, 95% CI 0.67–0.98 $p<0.03$

ANA, anastrozole; ATAC, Arimidex, Tamoxifen, Alone or in combination; BIG, Breast International Group; DFS, disease free survival; EXE, exemestane; LET, letrozole; LET, letrozole TAM, tamoxifen; TEAM, Tamoxifen, Exemestane Adjuvant Multicenter; TTDR, time to distant recurrence.

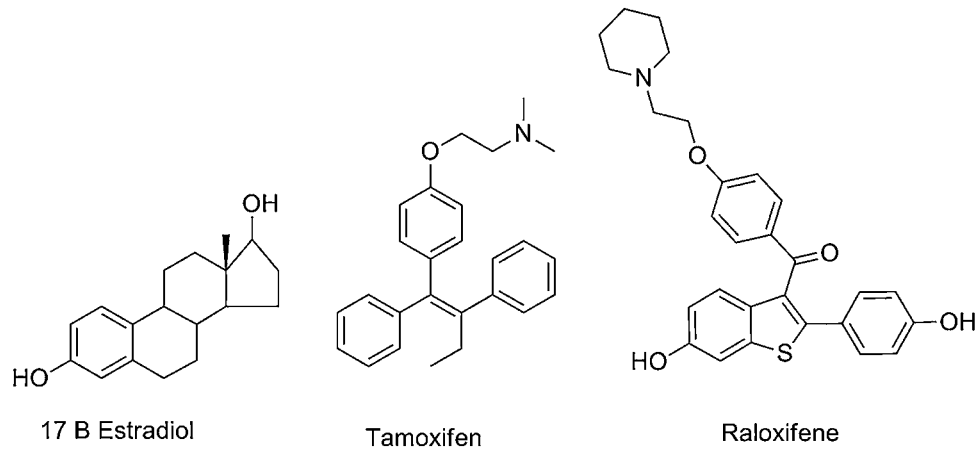


Fig. 1. Chemical Structures of SERMS currently used in breast cancer prevention. The structure of estradiol is included for comparison.

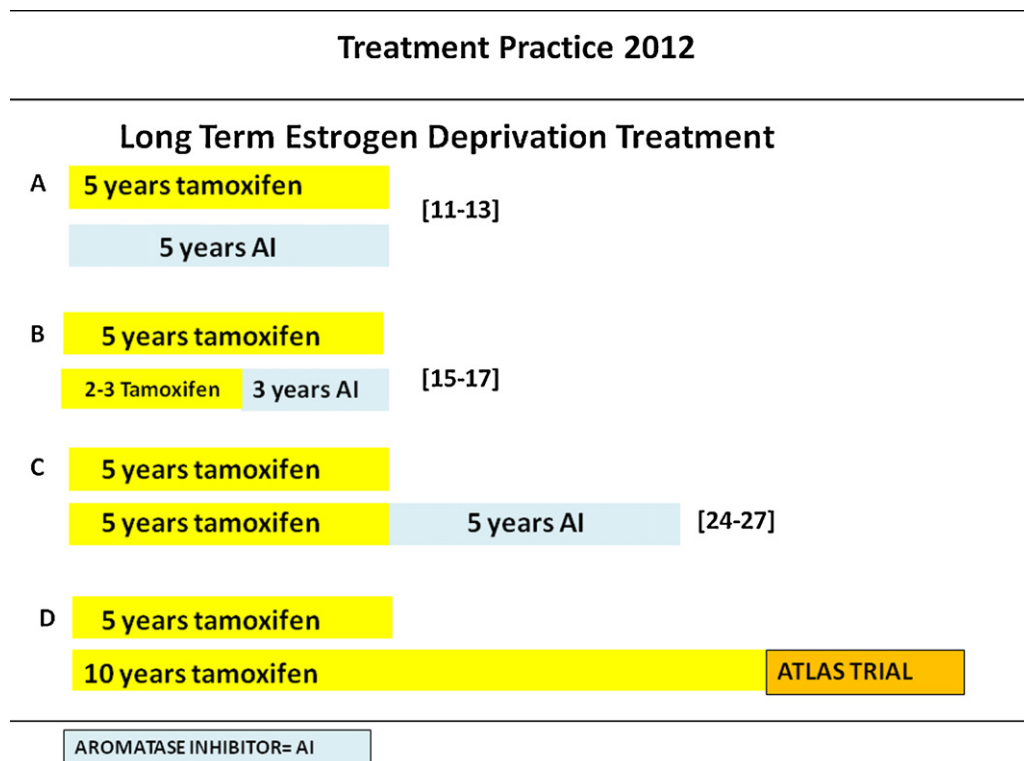


Fig. 2. Clinical guidelines in the adjuvant treatment of estrogen positive breast cancer in postmenopausal patients (A–C) or pre/postmenopausal patients (D). A. Five years of Tamoxifen or aromatase inhibitors can be used as first line adjuvant hormonal therapy in pre or perimenopausal or postmenopausal women respectively. B. In postmenopausal women, sequential therapy with aromatase inhibitor after 2–3 years of tamoxifen is comparable alternative to AI monotherapy. C. Additional 5 years with AIs after 5 years of tamoxifen, have shown significant disease free survival. D. Investigation of extension of tamoxifen beyond 5 years is presently ongoing.

response rate. To date no meaningful clinical differences have been demonstrated between third generation AIs.

2.4. Extended therapy

The MA-17 [25] randomly assigned 5187 patients who have completed 5 years of tamoxifen to 5 years of letrozole or placebo to determine the risk of recurrence. The study was stopped early when the first interim analysis showed that letrozole significantly lowered recurrence rate at a median follow up of 2.4 years. As a result the study was unblinded and 66% of patients on placebo crossed over to the letrozole group. An updated intent to treat analysis [26] revealed that letrozole treatment achieved a 2.9% improvement in DFS at 4 years (HR 0.68 $p=0.0001$). Similarly, ABCSG-6a [27] evaluated anastrozole for 3 years in comparison with placebo. Favorable results were obtained with anastrozole which resulted in a significant reduction in risk of recurrence ($p=0.031$). Exemestane was also compared with placebo after tamoxifen adjuvant therapy by the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-33. Similar to the MA-17, the study was stopped prematurely and unblinded due to significant improvement in DFS [28]. However all 3 extended adjuvant trials showed no significant improvement in overall survival. Although 10 year follow up of patients who received 5 years of tamoxifen yielded beneficial effects compared with 2 years of tamoxifen [29] extension of adjuvant therapy with tamoxifen beyond 5 years is not yet recommended. Results are currently awaited from the Adjuvant Tamoxifen-Longer Against Shorter (ATLAS) and adjuvant Tamoxifen Treatment offer more (aTTOM) which should give more insight to extending tamoxifen beyond 5 years. Furthermore, no data is available for the use of AIs beyond 5 years, therefore the recommended limit on AIs is 5 years total across strategies [21].

3. The SERM concept and breast cancer prevention

As a result of a focused effort to decipher the pharmacology and toxicology of tamoxifen, conclusions were built one upon the other, in the same laboratory, to define the properties of a new drug group called the SERMs and to articulation a roadmap to apply that drug group to prevent multiple diseases in women health. The mention of “modulation” at an ER target site occurred with the examination of the structure–function relationships of estrogenic triphenylethylene derivatives of tamoxifen at a prolactin gene target *in vitro* [30]. The estrogenic compounds could activate or suppress prolactin synthesis by altering the shape of the ER complex between the extremes of an “anti-estrogenic” or an “estrogenic” conformation [31]. This idea of the molecular modulation of the receptor at a single target site was then expanded to consider the physiologic responses that occurred with nonsteroidal antiestrogen at multiple target sites in the body simultaneously. A simultaneous series of translational studies focused on the uterus, breast (mammary gland) and bone together created the laboratory rationale for further clinical trials by the pharmaceutical industry [32–35]. It was clear in 1990 that the toxicological issues with tamoxifen e.g. endometrial cancer [35,36] needed another approach. A roadmap was stated because few women would have a prevention of breast cancer even in high risk populations; all would be exposed to side effects: “We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may as a side effect, significantly retard the development of breast cancer” [37].

Although tamoxifen is the pioneering SERM, raloxifene is the medicine that first exploited the “roadmap” successfully starting in 1992 [38]. Scientists [39] confirmed the concept in animal models measuring bone density, uterine weights and circulating cholesterol and initiated the Multiple Outcomes of Raloxifene Evaluation (MORE) trial. Raloxifene would be the first SERM to be approved for two of the three properties of the “ideal SERM”: reduction in the incidence of fractures in osteoporosis and the reduction in the incidence of breast cancer [40–42]. Raloxifene does not reduce the risk of coronary heart disease [43]. It is however, perhaps pertinent to note that the original work on the prevention of rat mammary carcinogenesis [34] concluded that because the pharmacokinetics of tamoxifen were superior to raloxifene then raloxifene was unlikely to be superior clinically in breast chemoprevention. Initially, data demonstrated that raloxifene was extremely effective at preventing ER positive breast cancer in 90% of osteoporotic women [41] but in the Study of Tamoxifen and Raloxifene or STAR trial in healthy postmenopausal women tamoxifen and raloxifene were equivalent in producing a 50% decrease in breast cancer incidence [42]. However, the latter evaluations were during treatment with the SERMs. If an evaluation of breast cancer incidence occurs after the end of a 5 year treatment regimen tamoxifen is superior to raloxifene that is only 78% as effective as tamoxifen 3 years following stopping treatment [44]. The laboratory study was accurate. As a result, continuous treatment with raloxifene can be considered and is efficacious at maintaining an antitumor environment [45]. Most importantly, there is no increased risk of endometrial cancer with raloxifene this again demonstrating the veracity of the translational research. Due to its breast cancer and osteoporosis preventive effects, raloxifene is recommended to be the ideal treatment of choice in high risk postmenopausal women.

4. New generation SERMS

The development of third generation SERMs was based on pre-clinical studies which showed beneficial estrogenic effects on the bone without the detrimental stimulation on the endometrium or breast tissue [46,47]. Lasofoxifene, Bazedoxifene, Arzoxifene and Ospemifene (Fig. 3) have been assessed in the treatment and prevention of osteoporosis as well as prevention of breast cancer. The Osteoporosis Prevention and Lipid Lowering (OPAL) and PEARL studies evaluated lasofoxifene, a third generation SERM in the treatment of osteoporosis. The OPAL study consists of two identical double-blind placebo-controlled studies assessing the vaginal and bone effects of lasofoxifene in nonosteoporotic women. Bone mineral density (BMD) was significantly reduced with an improvement in vaginal pH after 2 years of therapy [48–50]. The PEARL trial [51] is a randomized placebo controlled study involving 8556 postmenopausal women with low bone density. Five years treatment with 0.5 mg of lasofoxifene induced a significant 79% reduction of all breast cancers as well as a statistically significant reduction of vertebral (42%) and non vertebral fractures (24%), major coronary events (32%) and stroke (36%) when compared to placebo [52]. The CORAL trial [53] compared the effects of lasofoxifene, raloxifene and placebo on BMD of postmenopausal women. Although lasofoxifene and raloxifene had a similar adverse effect profile, lasofoxifene significantly improved lumbar spine BMD ($P \leq 0.05$), and significantly reduced low-density lipoprotein cholesterol levels ($P \leq 0.05$) at 2 years of therapy compared to raloxifene and placebo. Lasofoxifene was approved for the treatment of osteoporosis in the European Union in March 2009; however it is still under review by the FDA in the United States. The medicine has not been marketed.

A 2 year randomized double-blind study [54] assessed the clinical efficacy of bazedoxifene compared with placebo. Raloxifene was

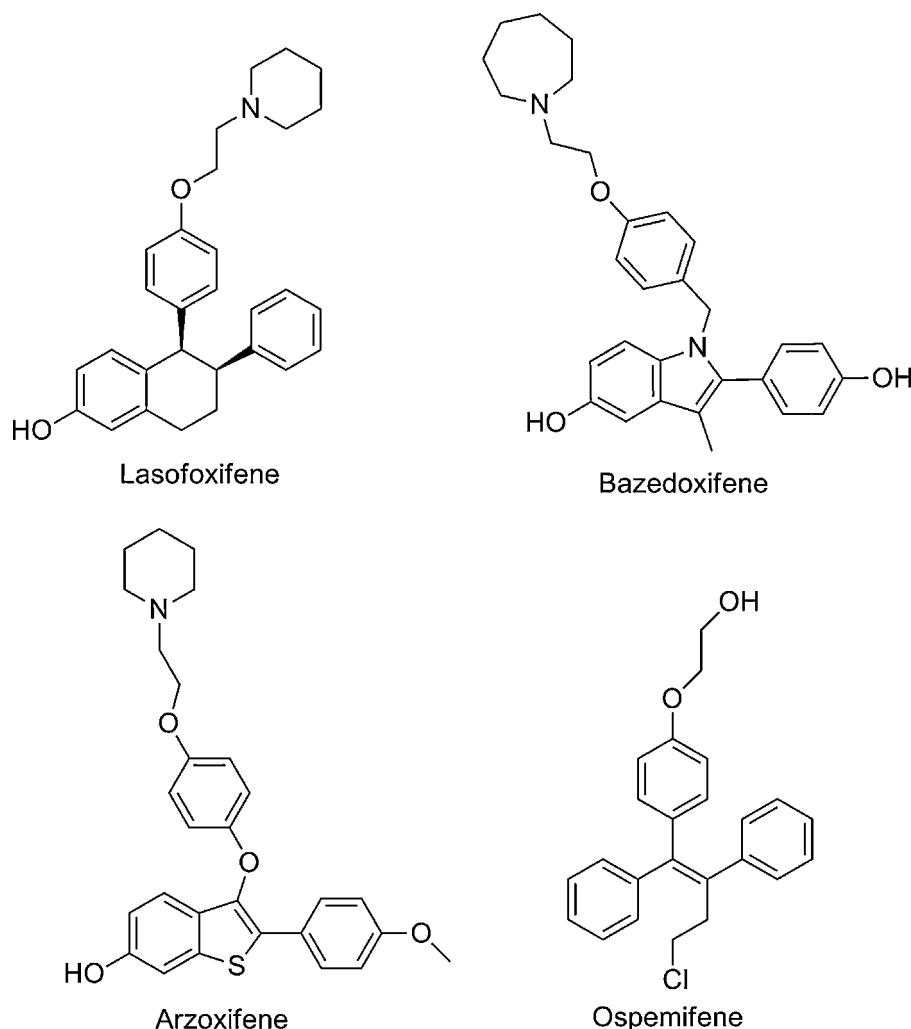


Fig. 3. Chemical structures of third generation SERMS.

added as a positive control. 10 mg, 20 mg and 40 mg of bazedoxifene had superior advantage over placebo in the improvement of BMD at all skeletal sites ($p < 0.001$). These effects were comparable to that obtained with 60 mg raloxifene. Incidence of cardiovascular disease, thromboembolic events, endometrial abnormalities and breast cancer did not significantly differ between treatment groups. Silverman and Colleagues [55] reported that the incidence of new vertebral fractures was significantly lower in the bazedoxifene group compared to placebo ($p < 0.05$), while incidence of non vertebral fractures was not statistically different from the placebo group at 3 years. This trend was maintained on extension of the study for an additional 2 years [56]. A post hoc analysis of a subgroup of women at higher fracture risk showed that bazedoxifene induced a 50% and 44% reduction in nonvertebral fracture risk relative to placebo ($p = 0.02$) and raloxifene ($p = 0.05$). This effect by bazedoxifene in comparison to placebo was supported by a re-analysis using the fracture probability tool, FRAX [57]. Although incidence of breast cancer was lower in the bazedoxifene group, there were no significant differences noted in the incidence of breast or endometrial carcinoma as well as endometrial hyperplasia among treatment groups. Because of the favorable outcomes seen with bazedoxifene on the endometrium and bone, the (Selective Estrogen Menopause and Response to Therapy) SMART-1 [58,59] trial investigated the combination of bazedoxifene (BZA) and conjugated estrogens (CE) compared to placebo using endometrial hyperplasia and BMD as the primary endpoints. Although treatment with

BZA/CE did not significantly reduce the incidence of endometrial hyperplasia over placebo at 2 years, BMD was increased significantly with BZA/CE at the lumbar spine and total hip. Perhaps the endometrial protective effects of BZA/CE may be seen with longer follow-up. This may alleviate the need for progestins in postmenopausal women with intact uterus on hormone replacement therapy. Bazedoxifene is currently approved for the treatment of osteoporosis in the European Union. Arzoxifene showed potential in the reduction of vertebral fractures but it was withdrawn from future clinical development based on nonvertebral efficacy [60]. Presently, FDA approval is being sought for the use of ospemifene in the treatment of vulvovaginal atrophy [61].

5. Conclusion

Tamoxifen continues to play a major role in the treatment and prevention of breast cancer. Parallel studies have shown that AIs are superior to tamoxifen in the management of metastatic breast cancer as well as an adjuvant agent in early breast cancer. Although most differences were statistically significant, however differences in overall survival was either non significant or was somewhat marginal. Clinical trials involving head to head comparison of AI are needed to determine the superiority (if any) in efficacy in tumor suppression. This will clarify the initial or sequential order in which these agents are used in clinical management. Tamoxifen and raloxifene are the only endocrine agents approved in the prevention

of breast cancer in high risk women. Newer SERMs, lasofoxifene and bazedoxifene are well tolerated agents and could possibly act as an alternative in the prevention of postmenopausal osteoporosis. These SERMs have shown comparable efficacy to raloxifene. However clinical validation is needed to confirm beneficial effects in the reduction of the incidence of breast cancer, cardiovascular and thromboembolic events. Progress with the new SERMs is currently dependent upon the financial advantages of new agents over old SERMs now as generics (tamoxifen) or ending their patent life (raloxifene). So what about no estrogen at all for chemoprevention? Two trials were established to evaluate the efficacy of anastrozole (IBISII trial) and exemestane (MAP.3) with placebo in the prevention of breast cancer in high risk postmenopausal women. Recently MAP.3 has demonstrated the value of reducing breast cancer incidence by a reported low incidence of side effects [62]. Nevertheless, AIs are not currently recommended for breast cancer risk reduction outside of a clinical trial. No other drugs have shown greater efficacy than those currently approved for breast cancer treatment and prevention.

In summary, it is reasonable to note that much progress has been made in women's health and a menu of medicines is now available and validated approaches are proven compared to none when all this started nearly 40 years ago [5].

Contributors

Ifeyinwa Obiorah MBBS, MSc: contribution: researched and wrote the article. V. Craig Jordan OBE, PhD, DSc, FMedSci: contribution: advised on referencing, diagrams and content.

Competing interests

The authors do not have any competing interests.

Funding

This work (VCJ) was supported by the Department of 1090 Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer 1095 Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

Provenance and peer review

Commissioned and externally peer reviewed.

Acknowledgments

This work (VCJ) was supported by the Department of 1090 Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen for the Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer 1095 Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

References

- Beatson G. On the treatment of inoperable cases of carcinoma of the mamma. Suggestions for a new method of treatment with illustrative cases. *Lancet* 1896;2:104–7.
- Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 2009;69:1243–54.
- Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol* 2006;147:S269–76.
- Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003;2:205–13.
- Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;44:30–8.
- Bonnerterre J, Buzdar A, Nabholz JM, et al. Anastrozole is superior to tamoxifen as first-line therapy in hormone receptor positive advanced breast carcinoma. *Cancer* 2001;92:2247–58.
- Mouridsen H, Gershanovich M, Sun Y, et al. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the International Letrozole Breast Cancer Group. *J Clin Oncol* 2003;21:2101–9.
- Paridaens RJ, Dirix LY, Beex LV, et al. Phase III study comparing exemestane with tamoxifen as first-line hormonal treatment of metastatic breast cancer in postmenopausal women: the European Organisation for Research and Treatment of Cancer Breast Cancer Cooperative Group. *J Clin Oncol* 2008;26:4883–90.
- Xu HB, Liu YJ, Li L. Aromatase inhibitor versus tamoxifen in postmenopausal woman with advanced breast cancer: a literature-based meta-analysis. *Clin Breast Cancer* 2011;11:246–51.
- Early Breast Cancer Trialists' Collaborative Group E. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 2011;378:771–84.
- Cuzick J, Sestak I, Baum M, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncol* 2010;11:1135–41.
- Mouridsen H, Giobbie-Hurder A, Goldhirsch A, et al. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. *N Engl J Med* 2009;361:766–76.
- Jones S, Seynaeve C, Hasenburger A, et al. Results of the first planned analysis of the TEAM (tamoxifen exemestane adjuvant multinational) prospective randomized phase III trial in hormone sensitive postmenopausal early breast cancer. *Cancer Res* 2009;69(Suppl.):15.
- Dowsett M, Cuzick J, Ingle J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* 2010;28:509–18.
- Jakesz R, Jonat W, Gnant M, et al. Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. *Lancet* 2005;366:455–62.
- Cuppone F, Bria E, Verma S, et al. Do adjuvant aromatase inhibitors increase the cardiovascular risk in postmenopausal women with early breast cancer. *Cancer* 2008;112:260–7.
- Boccardo F, Rubagotti A, Guglielmini P, et al. Switching to anastrozole versus continued tamoxifen treatment of early breast cancer. Updated results of the Italian tamoxifen anastrozole (ITA) trial. *Ann Oncol* 2006;17:vii10–4.
- Coombes RC, Kilburn LS, Snowdon CF, et al. Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. *Lancet* 2007;369:559–70.
- Kaufmann M, Jonat W, Hilfrich J, et al. Improved overall survival in postmenopausal women with early breast cancer after anastrozole initiated after treatment with tamoxifen compared with continued tamoxifen: the ARNO 95 Study. *J Clin Oncol* 2007;25:2664–70.
- van de Velde CJ, Rea D, Seynaeve C, et al. Adjuvant tamoxifen and exemestane in early breast cancer (TEAM): a randomised phase 3 trial. *Lancet* 2011;377:321–31.
- Burstein HJ, Prestrud AA, Seidenfeld J, et al. American Society of Clinical Oncology clinical practice guideline: update on adjuvant endocrine therapy for women with hormone receptor-positive breast cancer. *J Clin Oncol* 2010;28:3784–96.
- Geisler J, Helle H, Ekse D, et al. Letrozole is superior to anastrozole in suppressing breast cancer tissue and plasma estrogen levels. *Clin Cancer Res* 2008;14:6330–5.
- Rose C, Vtoraya O, Pluzanska A, et al. An open randomised trial of second-line endocrine therapy in advanced breast cancer: comparison of the aromatase inhibitors letrozole and anastrozole. *Eur J Cancer* 2003;39:2318–27.
- Ellis MJ, Suman VJ, Hoog J, et al. Randomized phase II neoadjuvant comparison between letrozole, anastrozole, and exemestane for postmenopausal women with estrogen receptor-rich stage 2–3 breast cancer: clinical and biomarker outcomes and predictive value of the baseline PAM50-based intrinsic subtype – ACOSOG Z1031. *J Clin Oncol* 2011;29:2342–9.
- Goss PE, Ingle JN, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early stage breast cancer. *N Engl J Med* 2003;349:1793–802.
- Ingle JN, Tu D, Pater JL, et al. Intent-to-treat analysis of the placebo-controlled trial of letrozole for extended adjuvant therapy in early breast cancer: NCIC CTG MA.17. *Ann Oncol* 2008;19:877–82.

- [27] Jakesz R, Greil R, Gnani M, et al. Extended adjuvant therapy with anastrozole among postmenopausal breast cancer patients: results from the randomized Austrian Breast and Colorectal Cancer Study Group Trial 6a. *J Natl Cancer Inst* 2007;99:1845–53.
- [28] Mamounas EP, Jeong JH, Wickerham DL, et al. Benefit from exemestane as extended adjuvant therapy after 5 years of adjuvant tamoxifen: intention-to-treat analysis of the National Surgical Adjuvant Breast And Bowel Project B-33 trial. *J Clin Oncol* 2008;26:1965–71.
- [29] Hackshaw A, Roughton M, Forsyth S, et al. Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. *J Clin Oncol* 2011;29:1657–63.
- [30] Jordan VC, Koch R, Mittal S, Schneider MR. Oestrogenic, antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. *Br J Pharmacol* 1986;87:217–23.
- [31] Lieberman ME, Gorski J, Jordan VC. An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 1983;258:4741–5.
- [32] Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;46:1870–4.
- [33] Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;10:31–5.
- [34] Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987;47:4020–4.
- [35] Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;48:812–5.
- [36] Fornander T, Rutqvist LE, Cedermark B, et al. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;1:117–20.
- [37] Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 1990;50:4177–89.
- [38] Lewis J, Jordan V. Comprehensive medicinal chemistry. In: Taylor J, Triggler D, editors. *Case Histories: Raloxifene*. Oxford Edition UK: Elsevier Limited; 2006, 103–121.
- [39] Black LJ, Sato M, Rowley ER, et al. Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. *J Clin Invest* 1994;93:63–9.
- [40] Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA* 1999;282:637–45.
- [41] Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of Raloxifene evaluation. *JAMA* 1999;281:2189–97.
- [42] Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 2006;295:2727–41.
- [43] Barrett-Connor E, Mosca L, Collins P, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med* 2006;355:125–37.
- [44] Vogel VG, Costantino JP, Wickerham DL, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 trial: preventing breast cancer. *Cancer Prev Res* 2010;3:696–706.
- [45] Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004;96:1751–61.
- [46] Komm BS, Lyttle CR. Developing a SERM: stringent preclinical selection criteria leading to an acceptable candidate (WAY-140424) for clinical evaluation. *Ann N Y Acad Sci* 2001;949:317–26.
- [47] Ke HZ, Paralkar VM, Grasser WA, et al. Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. *Endocrinology* 1998;139:2068–76.
- [48] McClung M, Siris E, Cummings S. Lasofoxifene increased BMD of the spine and hip and decreased bone turnover markers in postmenopausal women with low or normal BMD. *J Bone Miner Res* 2005;20:F429.
- [49] Davidson M, Moffett A, Welty F, et al. Extraskelletal effects of lasofoxifene on postmenopausal women. *J Bone Miner Res* 2005;20:S173.
- [50] Peterson GM, Naughton M, Tichelaar LK, Gennari L. Lasofoxifene: selective estrogen receptor modulator for the prevention and treatment of postmenopausal osteoporosis. *Ann Pharmacother* 2011;45:499–509.
- [51] Cummings SR, Ensrud K, Delmas PD, et al. Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med* 2010;362:686–96.
- [52] LaCroix AZ, Powles T, Osborne CK, et al. Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. *J Natl Cancer Inst* 2010;102:1706–15.
- [53] McClung MR, Siris E, Cummings S, et al. Prevention of bone loss in postmenopausal women treated with lasofoxifene compared with raloxifene. *Menopause* 2006;13:377–86.
- [54] Miller PD, Chines AA, Christiansen C, et al. Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. *J Bone Miner Res* 2008;23:525–35.
- [55] Silverman SL, Christiansen C, Genant HK, et al. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. *J Bone Miner Res* 2008;23:1923–34.
- [56] Silverman SL, Chines AA, Kendler DL, et al. Sustained efficacy and safety of bazedoxifene in preventing fractures in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled study. *Osteoporos Int* 2011. Epub. July 21. doi:10.1007/s00198-011-1691-1.
- [57] Kanis JA, Johansson H, Oden A, McCloskey EV. Bazedoxifene reduces vertebral and clinical fractures in postmenopausal women at high risk assessed with FRAX. *Bone* 2009;44:1049–54.
- [58] Pickar JH, Yeh IT, Bachmann G, Speroff L. Endometrial effects of a tissue selective estrogen complex containing bazedoxifene/conjugated estrogens as a menopausal therapy. *Fertil Steril* 2009;92:1018–24.
- [59] Lindsay R, Gallagher JC, Kagan R, et al. Efficacy of tissue-selective estrogen complex of bazedoxifene/conjugated estrogens for osteoporosis prevention in at-risk postmenopausal women. *Fertil Steril* 2009;92:1045–52.
- [60] Silverman SL. New selective estrogen receptor modulators (SERMs) in development. *Curr Osteoporos Rep* 2010;8:151–3.
- [61] Bachmann GA, Komi JO. Group aTOS. Ospemifene effectively treats vulvovaginal atrophy in postmenopausal women: results from a pivotal phase 3 study. *Menopause* 2010;17:480–6.
- [62] Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *N Engl J Med* 2011;364:2381–91.

Estrogen-Induced Apoptosis in Breast Cancer Cells: Translation to Clinical Relevance

Philipp Y. Maximov and Craig V. Jordan

*Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown,
University Medical Center, Washington, D.C.,
USA*

1. Introduction

The first example of hormonal dependency of breast cancer can be dated back as far as 1896, when Dr. G.T. Beatson observed and described the reduction of breast cancer progression in a premenopausal patient after bilateral oophorectomy (Beatson 1896). It was an indication that the ovaries produced something in a woman's body that fueled breast cancer growth. This phenomenon was reconfirmed in a collected series of patients with advanced breast cancer following oophorectomy (Boyd 1900), however there was only a 30% percent response. In 1916 Lathrop and Loeb demonstrated in mice, that ovarian function has an influence on the growth of mammary glands and tumorigenesis, and that castration of immature female mice has delayed the evolution of mammary tumors (Lathrop 1916). However, the chemical control mechanisms of breast cancer progression and the relevance of ovarian function remained uncertain, until the first animal models were introduced to test the effects of oophorectomy and estrogenic properties of different chemical compounds under precise laboratory conditions (Allen 1923). This model allowed the identification of the ovarian hormone, which induced estrus in oophorectomized mice, estrogen.

In subsequent years during the 1930s and 1940s many other compounds, including diethylstilbestrol, and triphenylethylene derivatives would be identified as estrogens utilizing the ovariectomized mouse model (Robson 1937; Dodds 1938). The connection between the beneficial effects of oophorectomy as a treatment for advanced breast cancer provoked questions about the actual role of estrogen and other estrogenic compounds in breast cancer growth. High dose estrogen therapy was the first chemical therapy ("chemotherapy") to treat any cancer successfully. In 1944 Haddow (Haddow 1944) published the results of his clinical trial with the synthetic estrogens triphenylchloroethylene, triphenylmethylethylene, and diethylstilbestrol. He found that 10 out of 22 post-menopausal patients with advanced mammary carcinomas, who were treated with triphenylchloroethylene, had significant regression of tumor growth. Five patients out of 14 who were treated with high dose stilbestrol produced similar responses. The finding that high doses of synthetic estrogens induced regression of tumor growth in some, but not all postmenopausal patients with breast cancer (30% of patients responded to therapy favorably) was similar to the random responsiveness of oophorectomy in premenopausal patients with metastatic breast cancer (Boyd 1900). However, Haddow (Haddow 1944) noted that the first successful use of a chemical therapy to treat breast and prostate cancers

was affiliated with significant systemic side effects, such as nausea, areola pigmentation, uterine bleeding, and edema of the lower extremities. At approximately same time Walpole was investigating the role of diethylstilbestrol and dienestrol in breast cancer (Walpole 1948). He confirmed the results obtained by Haddow that estrogens are effective in the treatment of breast cancer and can be of benefit for patients, but also noticed that older women, and women who received higher doses of estrogens had a better response to hormonal therapy (Walpole 1948; Haddow 1950). However, the mechanisms were again undefined.

The first successful attempt to decipher the biochemistry of estrogens in mammals occurred a decade later. Tritium-labeled hexestrol was found to accumulate in reproductive organs, including mammary glands, in female goats and sheep (Glascok and Hoekstra 1959). This finding was a crucial observation to understand the role of estrogens in processes involving target tissues, such as the mammary gland. Subsequently this research was translated to the clinic with the finding that tritium-labeled hexestrol accumulated at a higher rate in patients that favorably respond to adrenalectomy and oophorectomy, comparing to patients that do not (Folca et al. 1961). This indicated that patients who would accumulate estrogens better in target breast tissue would respond better to surgical castration. However, this technical approach was not pursued further.

During the 1950's Kennedy (Kennedy and Nathanson 1953) systematically investigated the efficacy of synthetic estrogens for the treatment of advanced breast cancer. Kennedy examined a variety of different estrogens, however he found no significant differences and diethylstilbestrol became the standard drug. However, side effects still remained a concern and responses lasted for only about a year in the majority of patients. By the 1960's, the standards for the hormonal treatment of breast cancer were established. Premenopausal women were to be treated with ovarian irradiation therapy or bilateral oophorectomy. However, based on data from the clinical trials, postmenopausal patients with advanced breast cancer were to be treated with high dose of the most potent synthetic estrogenic compound diethylstilboestrol (Kennedy 1965). Overall, one could anticipate that 36 % of patients would respond favorably to high dose estrogen therapy (Kennedy 1965). However, the molecular mechanisms of the anticancer action of estrogen remained elusive. In 1970 Haddow (Haddow 1970) was not enthusiastic about the overall prospects of chemical therapy of breast cancer, he felt that it was important that safer less toxic "estrogens" were developed that might extend therapeutic use. There were clues that deciphering the mysteries of endocrine therapy, such as unknown mechanisms of tumor regression after high-dose estrogen therapy, which could be of major benefit for patient's treatment. Haddow stated: "In spite of the extremely limited practicality of such measure [high dose estrogen], the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases has always been regarded as of major theoretical importance, and it is a matter of some disappointment that so much of the underlying mechanisms continues to elude us". However, as noted previously, high dose estrogen therapy was more successful as a treatment for breast cancer the farther the woman was from the menopause. Estrogen withdrawal somehow played a role in sensitizing tumors to the antitumor actions of estrogen, but this fact was not appreciated at that time. We will return to this concept.

Elwood Jensen predicted the existence of estrogen receptor (ER) in 1962 (Jensen 1962), and the isolation and identification of the ER protein by Toft and Gorski occurred in 1966 (Toft and Gorski 1966). The mediating role of the ER in the estrogen responsiveness of breast

cancer was established, and eventually the ER became the molecular target for targeted therapy and prevention of ER-positive breast cancer (Jensen and Jordan 2003). It was suggested (Lacassagne 1936) in 1936 that a therapeutic agent to block estrogen action would be useful in breast cancer prevention, but there were no clues. Potential candidate antiestrogens were only discovered 20 years later in the late 1950s, but these agents were identified and screened as contraceptive drugs in laboratory animals. MER25 (Lerner et al. 1958), which was first reported as a non-steroidal antiestrogen and subsequently found to be a post-coital contraceptive in animals (Lerner and Jordan 1990). But the drug was too toxic. The first clinically useful compound MRL41 or clomiphene was tested in women; however, it was not a contraceptive, but actually induced ovulation. Nevertheless, clinical trials of clomiphene in the early 1960's did move forward to evaluate its activity in the treatment of breast cancer, but were terminated because of concerns about the drug's potential to cause cataracts (Jordan 2003). In parallel studies stimulated by the initial reports of the non-steroidal antiestrogens, ICI 46,474, the pure trans-isomer of a substituted triphenylethylene, was discovered at Imperial Chemicals Industry (ICI) Pharmaceuticals (now Astra Zeneca) and was described as a postcoital contraceptive in the rat (Harper and Walpole 1967). The Head of the Fertility Control program, Arthur Walpole, earlier in his career was interested in why only some postmenopausal women with metastatic breast cancer respond favorably to high dose estrogen therapy (Walpole 1948). Later Walpole ensured that ICI 46,474 was tested in the clinic and placed on the market as an orphan drug while ICI invested in the scientific research by others in academia to conduct a systematic study of the anticancer actions of tamoxifen and its metabolites (Jordan 2008). This investment reinvented tamoxifen as the first anticancer agent specifically targeted to the ER in the tumor and created the scientific principles to ultimately establish tamoxifen as the "gold standard" for the adjuvant therapy of breast cancer and as the first chemopreventative agent that reduces the incidence of breast cancer in women with elevated risk (Fisher et al. 1999; EBCTCG 2005).

2. Development and clinical application of antihormonal therapy

Since the clinical application of the laboratory principle of targeting the ER with long-term antihormonal therapy (Jordan 2008) to treat breast cancer has become the standard of care, two different approaches to adjuvant antihormonal therapy have been developed in the past 30 years: first, is the blockade of estrogen-stimulated growth (Jensen and Jordan 2003) at the tumor ERs with antiestrogens, and the second one, is the use of aromatase inhibitors to block estrogen biosynthesis in postmenopausal patients (Jordan and Brodie 2007). Tamoxifen was originally referred to as a non-steroidal antiestrogen (Harper and Walpole 1967). However, as more has become known about its molecular pharmacology (Jordan 2001) it has become the pioneering Selective Estrogen Receptor Modulator (SERM). The concept of SERM action was defined by four main pieces of laboratory evidence: 1) ER-positive breast cancer cells inoculated into athymic mice grew into tumors in response to estradiol, but not to tamoxifen (antiestrogenic action), however both estradiol and tamoxifen induced uterine weight increase in mice (estrogen action) (Jordan and Robinson 1987); 2) raloxifene (another non-steroidal antiestrogen), which is less estrogenic in rat uterus, maintained the bone density in ovariectomized rats (estrogen action), as did tamoxifen (Jordan et al. 1987), and prevented mammary carcinogenesis (antiestrogenic action) (Gottardis and Jordan 1987); 3) tamoxifen blocked estradiol-induced growth of ER-positive breast cancer cells in athymic mice

(antiestrogenic action), but induced rapid growth of ER-positive endometrial carcinomas (estrogenic action) (Gottardis et al. 1988); 4) raloxifene was less effective in promoting endometrial cancer growth than tamoxifen (less estrogenic action in uterine tissue) (Gottardis et al. 1990). These laboratory results all translated into clinical practice where it was shown that tamoxifen effectively can reduce the incidence of breast cancer in high-risk pre- and postmenopausal women, however increases the incidence of blood clots and endometrial cancer, which is linked to estrogen-like actions of tamoxifen in these tissues in postmenopausal women, who have a low-estrogen environment (Fisher et al. 1998).

Aromatase inhibitors have an advantage in the therapy of postmenopausal patients over tamoxifen, firstly, because there are fewer side effects, such as blood clots or endometrial cancer, and aromatase inhibitors have a small, but still significant efficacy in increasing disease free survival (Howell et al. 2005). However, most postmenopausal patients worldwide continue treatment with tamoxifen, either for economic reasons or because they were hysterectomized and also have a low risk of developing blood clots (low body mass index and are athletically active). In premenopausal women, long term tamoxifen is the antihormonal therapy of choice for the treatment of ductal carcinoma in situ (DCIS) (Fisher et al. 1999), ER-positive breast cancer treatment (EBCTCG 2005) and the reduction of breast cancer incidence in those premenopausal women at elevated risk (Fisher et al. 1998). It is important to stress that premenopausal women treated with tamoxifen do not have elevations in endometrial cancer and blood clots, thus risk: benefit ratio is in favor of tamoxifen treatment (Gail et al. 1999).

The development of raloxifene from a laboratory concept (Jordan 2007) to a clinically effective drug to prevent both osteoporosis and breast cancer (Cummings et al. 1999; Vogel et al. 2006) has created new opportunities for clinical applications of SERMs. Raloxifene is the result. However, the biggest advantage of raloxifene is that it does not increase the incidence of endometrial cancer (Vogel et al. 2006), which was noted in postmenopausal women taking tamoxifen (Fisher et al. 1998). Raloxifene is used primarily for the prevention of osteoporosis and for the prevention of breast cancer in high risk postmenopausal women. The current clinical trend for the use of antihormonal therapy for the treatment and prevention of breast cancer is to employ long-term treatment durations. Currently aromatase inhibitors are used for a full 5 years after 5 years of tamoxifen (Goss et al. 2005). Though, the clinical application of the SERM concept has proven itself to be successful for the prevention of osteoporosis and 50% of breast cancers (Vogel et al. 2006; Vogel et al. 2010), drug resistance remains an important issue arising from long-term SERM treatment. Studies have shown that after long-term SERM treatment, the pharmacology of the SERMs changes from an inhibitory antiestrogenic state to a stimulatory estrogen-like response (Gottardis and Jordan 1988).

3. Evolution of SERM resistance as deciphered by the laboratory models

Clinical and laboratory studies have identified possible mechanisms for the acquired resistance to SERMs, and tamoxifen. Acquired resistance to SERMs is unique as the tumors are SERM stimulated for growth (Howell et al. 1992). The first laboratory model (Gottardis and Jordan 1988; Gottardis et al. 1988; Gottardis et al. 1990) of transplantable tamoxifen resistant cells demonstrated that 1) tamoxifen or estrogen can cause tumors to grow, 2) tumors require a liganded receptor to grow, 3) an aromatase inhibitors (estrogen deprivation) or a pure antiestrogen that causes ER degradation would be useful second line

agents, 4) there was cross resistance with other SERMs (O'Regan et al. 2002). Currently, numerous model systems exist to study SERM resistance. Some are engineered to increase the likelihood of resistance (Osborne et al. 2003) and others are engineered by transfection of the aromatase gene to study resistance to aromatase inhibitors and compare them with tamoxifen (Brodie et al. 2003). In contrast, others have chosen to develop models naturally through selective pressure either *in vivo* or *in vitro*. The natural selection approach is to either continuously transplant the resulting SERM resistant breast cancer into SERM-treated athymic animals (Wolf and Jordan 1993; Lee et al. 2000) or to employ strategies *in vitro* that use continuous SERM treatment (Herman and Katzenellenbogen 1996; Liu et al. 2003; Park et al. 2005) or long term estrogen deprivation in culture (Song et al. 2001; Lewis et al. 2005). Distinct phases of resistance were elucidated with the use of unique models of tamoxifen-resistant breast cancer developed *in vivo*, in order to better understand the biological consequences of extended antiestrogen treatment on the survival of breast cancer. The model for the treatment phase was developed by injecting ER α -positive MCF-7 cells into athymic mice and supplementing them with post-menopausal doses of estradiol (E2) (86–93 pg/ml) (Robinson and Jordan 1989), which were estradiol-stimulated and tamoxifen (TAM)-inhibited (Figure 1).

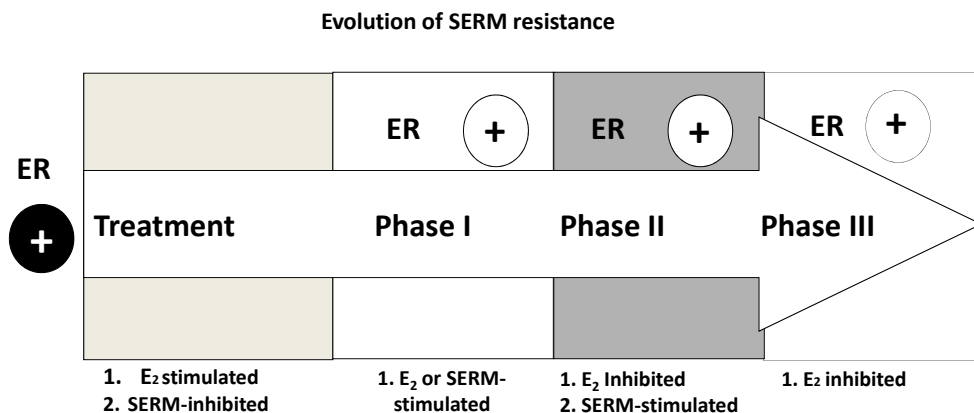


Fig. 1. Evolution of SERM resistance as observed in animal models.

With short term treatment (<2 years) with tamoxifen Phase I TAM-resistant breast tumors developed, which were stimulated to grow by both E2 and tamoxifen (Figure 1) (Gottardis and Jordan 1988; Osborne et al. 1991). The novel model of Phase II resistance to tamoxifen was developed by long-term treatment (>5 years) of breast tumors with tamoxifen (MCF-7TAMLT). These MCF-7TAMLT tumors were stimulated to grow with tamoxifen, but paradoxically were inhibited by estradiol (Figure 1) (Wolf and Jordan 1993; Yao et al. 2000; Osipo et al. 2003). The phase when all known therapies fail and only E2-inhibit the growth is referred to as phase III resistance (Figure 1) (Jordan 2004). Interestingly, during the progression from the treatment phase to Phase III resistance, a cyclic phenomenon was observed where initially estradiol-inhibited growth of Phase II TAM-resistant tumors followed by re-sensitization to estradiol as a growth stimulant (Yao et al. 2000). These new estradiol-stimulated MCF-7 tumors from Phase II tamoxifen-resistant tumors were inhibited by treatment with either TAM or fulvestrant demonstrating complete reversal of drug resistance to tamoxifen (Yao et al. 2000). A similar phenomenon was observed with

raloxifen-resistance (Balaburski et al. 2010). In addition to SERM-resistant tumors, estradiol, at physiologic concentrations, has also been shown to induce apoptosis in long term estrogen deprived (LTED) breast cancer cells *in vitro* and *in vivo*. We noted previously, that in the past, pharmacologic estrogen was employed in therapy of advanced breast cancer that resulted in favorable responses with regression of disease (Haddow 1944). Estrogen therapy yields as high as 40% response rate as first-line treatment in patients with hormonally sensitive breast cancer with metastatic disease (Ingle et al. 1981) and approximately 31% in patients heavily pre-treated with previous endocrine therapies (Lonning et al. 2001). The unique aspect of current laboratory findings is that physiologic estrogen can induce tumor regression in long-term anti-hormone drug resistance (Wolf and Jordan 1993; Yao et al. 2000; Song et al. 2001; Jordan and Ford 2011). But what are the mechanisms?

Known mechanisms of estrogen-induced apoptosis in LTED breast cancer cells

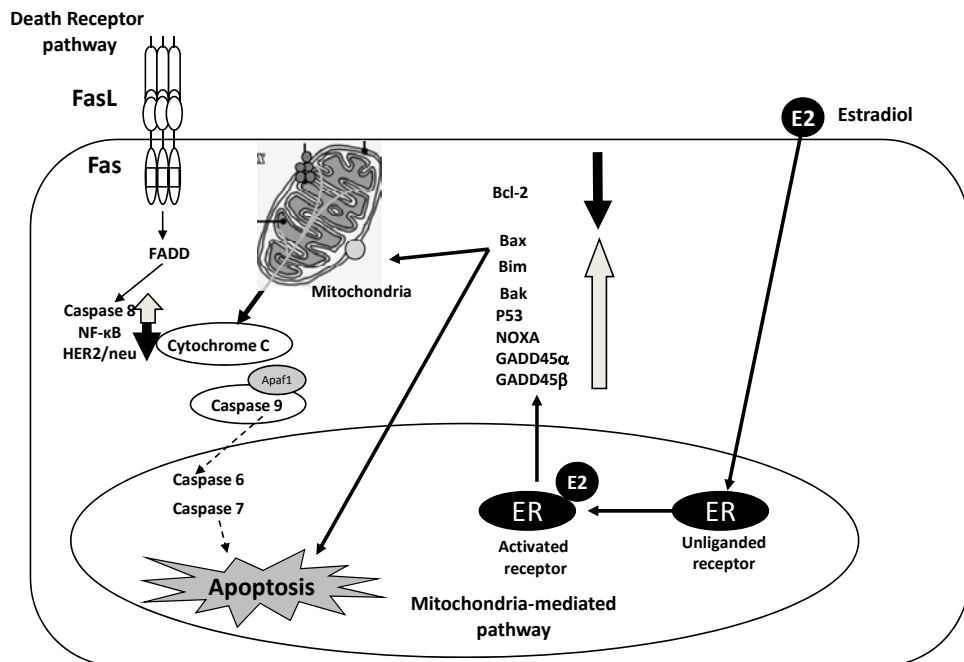


Fig. 2. Mechanisms of estrogen-induced apoptosis in Long-Term Estrogen Deprived (LTED) breast cancer cells. Both FasR/FasL death-signaling and mitochondrial pathways are involved.

4. Mechanism of estrogen-induced apoptosis

To investigate the mechanisms of estradiol-induced apoptosis SERM-stimulated models (Liu et al. 2003; Osipo et al. 2003) or long-term estrogen deprived MCF-7 breast cancer cell lines (Song et al. 2001; Lewis et al. 2005; Lewis et al. 2005) have been interrogated. A link between estradiol-induced apoptosis and activation of the FasR/FasL death-signaling pathway was demonstrated in tamoxifen-stimulated breast cancer tumors by inducing the death receptor

Fas with physiologic levels of estradiol and suppressing the antiapoptotic/prosurvival factors NF- κ B and HER2/neu (Osipo et al. 2003; Lewis et al. 2005). A similar finding was reported (Liu et al. 2003) for raloxifene-resistant tumor cells where the growth of raloxifene-resistant MCF-7/Ral cells *in vitro* and *in vivo* was repressed by estradiol via mechanism involving increased Fas expression and decreased NF- κ B activity. Furthermore, MCF-7 cells deprived of estrogen for up to 24 months (MCF-7LTED) *in vitro* expressed high levels of Fas compared to the parental MCF-7 cells, which do not express Fas and treatment of the MCF-7/LTED cells with estradiol resulted in a marked increase in Fas ligand (FasL) in these cells (Song et al. 2001). It was also noted that mitochondrial pathway could play a role in mediating estrogen induced apoptosis as the basal expression levels of Bcl-2 were higher in these cells than in the parental MCF-7 cells. Estradiol induced apoptosis occurs in a LTED breast cancer cell line named MCF-7:5C by neutralization of the Bcl-2/Bcl-XL proteins, and upregulation of proapoptotic proteins such as Bax, Bak and Bim, which proves the role of intrinsic mitochondrial pathway (Lewis et al. 2005) (Figure 2).

In MCF-7:5C cells the expression of several pro-apoptotic proteins—including Bax, Bak, Bim, Noxa, Puma, and p53—are markedly increased with estradiol treatment and blockade of Bax and Bim expression using siRNAs almost completely reversed the apoptotic effect of estradiol. Estradiol treatment also led to a loss of mitochondrial potential and a dramatic increase in the release of cytochrome *c* from the mitochondria, which resulted in activation of caspases and cleavage of PARP. Furthermore, overexpression of anti-apoptotic Bcl-XL was able to protect MCF-7:5C cells from estradiol-induced apoptosis. This particular study was the first to show a link between estradiol-induced cell death and activation of the mitochondrial apoptotic pathway using a breast cancer cell model resistant to estrogen withdrawal (Lewis et al. 2005). Besides the action on the mitochondrial pathway, Bcl-2 overexpression increases cellular glutathione (GSH) level which is associated with increased resistance to chemotherapy-induced apoptosis (Voehringer 1999). GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. It is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent intracellular antioxidant protecting cells from toxins such as free radicals (Schroder et al. 1996; Anderson et al. 1999). Changes in GSH homeostasis have been implicated in the etiology and progression of some diseases and breast cancer (Townsend et al. 2003) and studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis (Anderson et al. 1999). Our laboratory has found evidence which suggests that GSH participates in retarding apoptosis in antihormone-resistant MCF-7:2A human breast cancer cells, which have ~60% elevated levels of GSH compared to wild-type MCF-7 cells and unable to undergo estrogen-induced apoptosis within 1 week unlike MCF-7:5C cells, and that depletion of GSH by 100 μ M of L-buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis, sensitizes these resistant cells to estradiol-induced apoptosis (Lewis-Wambi et al. 2008). However, the question arises as to the actual mechanism of the apoptotic trigger mediated by the ER complex.

5. Structure-function relationship studies for deciphering estrogen-induced apoptosis

The fact that SERMs do not affect the spontaneous growth of MCF-7:5C cells, but can completely block estradiol-induced apoptosis, was an important clue that the shape of the

ER can be modulated to prevent apoptosis. Extensive structure-function relationship studies were initially used to develop a molecular model of estrogen and antiestrogen action (Lieberman et al. 1983; Jordan et al. 1984; Jordan et al. 1986). The hypothetical model presumed the envelopment of a planar estrogen within the ligand-binding domain (LBD) of the ER complex. In contrast, the three-dimensional triphenylethylene binding in the LBD cavity prevents full ER's activation by keeping the LBD open. This structural perturbation of the ER complex is achieved by a correctly positioned bulky side chain on the SERM. This model was enhanced by the subsequent studies to solve the X-ray crystallography of the LBD ER's bound with an estrogen or an antiestrogen (Brzozowski et al. 1997; Shiau et al. 1998). The LBD of ER α is formed by H2-H11 helices and the hairpin β -sheet, while H12, in the agonist bound conformation closes over the LBD cavity filled with E2. E2 is aligned in the cavity by hydrogen bonds at both ends of the ligand, particularly the 3-OH group at the A-ring end of E2. This allows hydrophobic van der Waals contacts along the lipophilic rings of E2, in particular between Phe404 and E2's A-ring, to promote a low energy conformation (Brzozowski et al. 1997). This results in sealing of the ligand-binding cavity by H12, and exposes the AF-2 motif at the surface of the receptor for interaction with coactivators to promote transcriptional transactivation. In contrast, 4-hydroxytamoxifen binds to ER's LBD to block the closure of the cavity by relocating H12 away from the binding pocket, thus preventing coactivator molecules from binding to the appropriate site on the external surface of the complex, which produces an antiestrogenic effect (Shiau et al. 1998). Therefore, it is the external shape of the ERs that is being modulated by the ligand which dictates the binding of coactivator molecules. In other words, the shape of the ligand actually causes the receptor to change shape and programs the ER complex to be able to bind coregulator molecules. However, the simple model of a coregulator controlling the biology of an ER complex is not that simple. The modulation of the estrogen target gene is in fact, regulated by a dynamic process of assembly and destruction of transcription complex at the promoter site of a target gene. After ER is bound to an agonist ligand, its conformation changes allowing coregulator molecules to bind to the complex, for example, SRC-3. SRC-3 is a core coactivator that also attracts other coregulators that do not directly bind to ER, such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases UbC and UBL. All of these coregulators perform specific subreactions within the protein complex of ER and DNA necessary for transcription of target genes, such as chromatin remodeling through methylation and acetylation modifications, and also direct their enzymatic activity towards adjacent factors, which promote dissociation of the coactivator complex and subsequent ubiquitination of select components for proteosomal degradation. As a result, this allows the next cycle of coactivator-receptor-DNA interactions to proceed and the binding and degradation of transcription complexes sustaining the gene transcription (Lonard et al. 2000). However, although AF-2 is deactivated by 4OHTAM, the 4OHTAM:ER α complex has estrogen-like activity (Levenson et al. 1998), whereas raloxifene does not (Levenson et al. 1997). This is believed to be because the side chain of raloxifene shields and neutralizes asp351 to block estrogen action (Levenson and Jordan 1998). In contrast the side chain of tamoxifen is too short. It appears that when helix 12 is not positioned correctly the exposed asp351 can interact with AF-1 to produce estrogen action. This estrogen-like activity can be inhibited by substituting asp351 for glycine an uncharged amino acid (MacGregor Schafer et al. 2000). However, knowledge of the structure of the

4OHTAM: ER LBD complex (Shiau et al. 1998) led to the idea that all estrogens may not be the same in their interactions with ER (Jordan et al. 2001). Previous studies suggest that non-planar TPEs with a bulky phenyl substituent prevents helix-12 from completely sealing the LBD pocket (Jordan et al. 2001). This physical event creates a putative 'anti-estrogen like' configuration within the complex. However, the complex is not anti-estrogenic because Asp351 is exposed to communicate with AF-1 thus causing estrogen-like action. Therefore, there are putative Class I (planar) and Class II (non-planar) estrogens (Jordan et al. 2001). A similar classification and conclusion has been proposed (Gust et al. 2001), but the biological consequences of this classification were unknown until recently.

To further address the hypothesis that the shape of the ER complex can be controlled by the shape of an estrogen, and thereby altering its functional properties, such as induction of apoptosis, a range of hydroxylated TPEs was synthesized (Figure 3) to establish new tools to investigate the relationship of shape with estrogenic activity through the exposure of asp351 (Maximov et al. 2010).

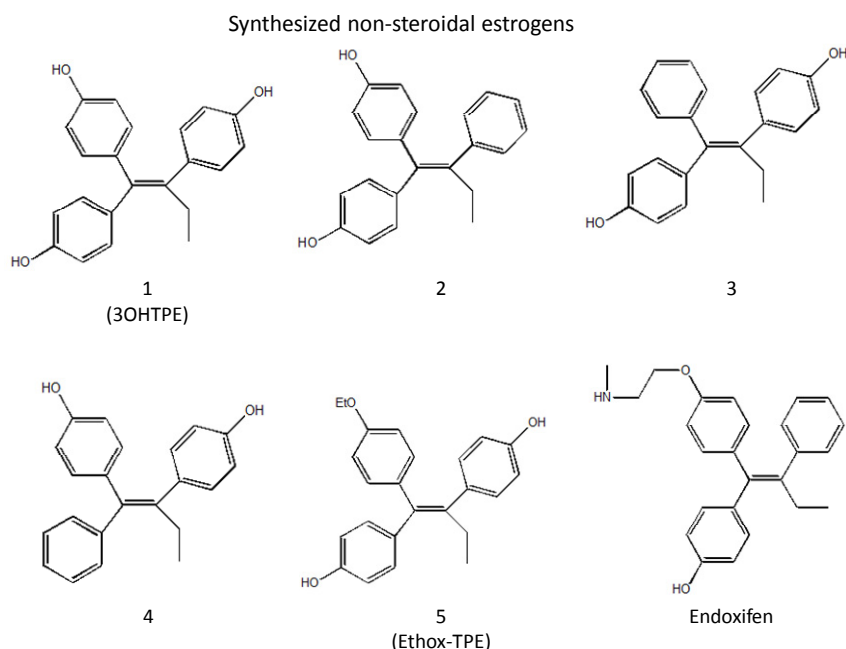


Fig. 3. Synthesized class II non-steroidal estrogens. All estrogens are hydroxylated derivatives of triphenylethylene; 1 – 3-hydroxytriphenylethylene (3OHTPE), 2- bisphenoltriphenylethylene, 3 – E-dihydroxytriphenylethylene, 4- Z-dihydroxytriphenylethylene, 5- ethoxytriphenylethylene, and Endoxifen (a metabolite of the antiestrogenic triphenylethylene tamoxifen with high affinity for the estrogen receptor).

We compared and contrasted the estrogen-like properties of the hydroxylated TPEs to promote proliferation in the ER α -positive human breast cancer cell line MCF-7:WS8 cells (Figure 4A), which are hypersensitive to the proliferative actions of E2. Compounds were compared with the tamoxifen metabolites 4-OHT and endoxifen. Results show that our

MCF-7:WS8 human breast cancer cells were exquisitely sensitive to E2 which produced a concentration-dependent increase in growth, and all of the TPE's were potent agonists with the ability to stimulate MCF-7:WS8 breast cancer cell growth, however, their agonist potency was less compared to E2. The metabolites, 4-OHT and endoxifen, had no significant agonist effect in MCF-7:WS8 cells, however, these compounds at 1 μ M were able to completely inhibit estradiol-stimulated MCF-7:WS8 breast cancer cell growth, thus confirming their role as antiestrogens (data not shown). To determine the ability of the test TPEs to activate the ER, MCF-7:WS8 cells were transiently transfected with an ERE-luciferase reporter gene encoding the firefly reporter gene with 5 consecutive Estrogen Responsive Elements (EREs) under the control of a TATA promoter. The binding of ligand-activated ER complex at the EREs in the promoter of the luciferase gene activates transcription. The measurement of the luciferase expression levels permits a determination of agonist activity of the TPE:ER complex. All the phenolic TPEs were estrogenic and induced the increase of ERE-luciferase activity, but were less potent compared to E2. To confirm and advance the hypothesis that the shape of the estrogen ER complex was different for planar and nonplanar (TPE -like) estrogens, series of tested phenolic TPEs were evaluated in the ER-negative breast cancer cell line T47D:C42 (Pink et al. 1996) which was transiently transfected with an ERE luciferase plasmid and either the wild-type ER or the D351G mutant ER plasmids. Previously it was found that the mutant D351G ER completely suppressed estrogen-like properties of 4-OHT at an endogenous TGF α target gene (MacGregor Schafer et al. 2000). We established that in the presence of the wild-type ER all of the tested TPE compounds were potent agonists with the ability to significantly enhance ERE luciferase activity (Figure 4C). In contrast, when the D351G mutant ER gene was transfected with the ERE luciferase reporter only the planar E₂ was estrogenic whereas the TPEs did not activate the ERE reporter gene (Figure 4D). These results confirm the importance of Asp351 in ER activation by TPE ligands to trigger estrogen action. To further confirm the hypothesis, the best "fits" of the tested TPEs and endoxifen, obtained from docking simulations ran against the antagonist conformation of the ER, were superimposed on the experimental agonist conformation of the ER. Overall the TPEs are unlikely to be accommodated in the agonist conformation of the ER due to the sterical clashes between "Leu crown", mostly Leu525 and Leu540, helix 12 and ligands, indicating, that these ligands most likely bind to ER's conformation more closely related with the antagonist form. X-ray crystallography of ER-4OHTAM and ER-Raloxifene complexes, demonstrating that the presence of the alkylaminoethoxy sidechain of 4OHTAM is crucial for the ER to gain an antagonistic conformation by displacing the H12 of the receptor by 4OHTAM's bulky sidechain, thus preventing the binding of the coactivators (Shiau et al. 1998). The absence of the alkylaminoethoxy sidechain on the tested TPEs does not allow these compounds to act as antiestrogens, like 4-OHT or endoxifen, which possesses the alkylaminoethoxy sidechain (Shiau et al. 1998). However, the fact that these TPEs were able to significantly induce growth and ERE activation in MCF-7:WS8 cells demonstrated that they are still full agonists, despite the changes in biological potencies of the tested TPEs, due to repositioning of the hydroxyl groups and addition of the ethoxy group. Thus cell growth is a very sensitive property of the ligand:ER complex and can occur minimally with an AF-1 function alone in the case of TPEs but also with the possibility for interacting with a perturbed LBD. 4OHT does not stimulate growth so possibly a corepressor binds in the case of a SERM:ER complex. An interesting aspect of the study (Maximov et al. 2010) is the importance of Asp351 in activation of the ER thereby acting as a molecular test for the presumed structure

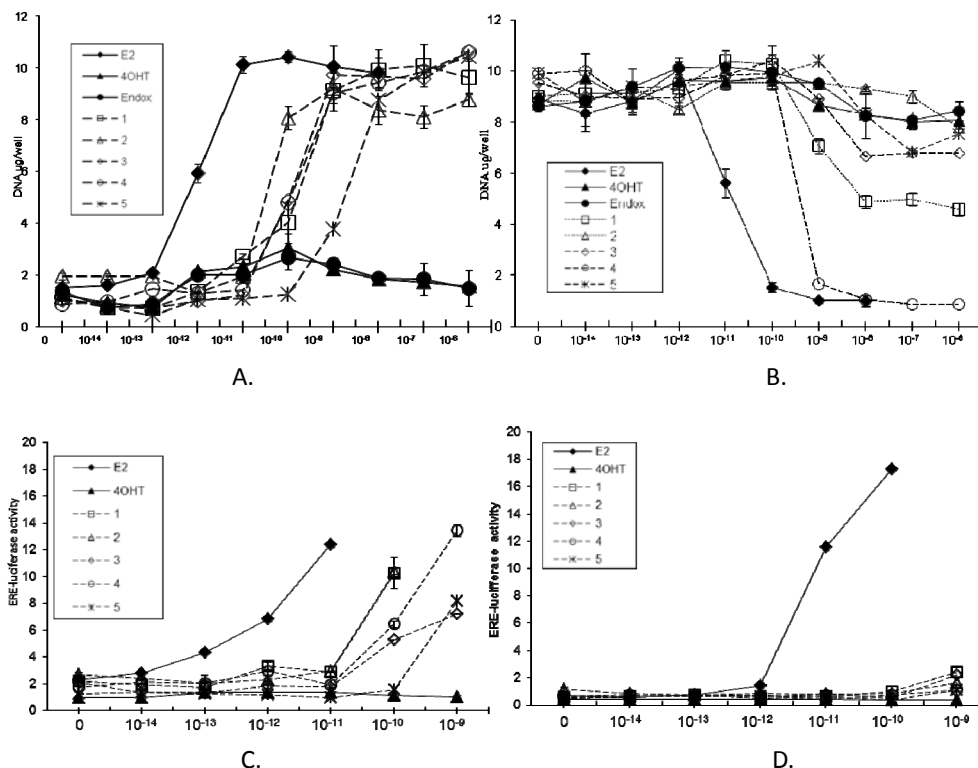


Fig. 4. A: Agonist activity in MCF-7:WS8 cells of synthesized TPEs and E2 and anti-estrogens 4-OHT and Endoxifen; B: E2 induces apoptosis in long-term estrogen deprived MCF-7:5C cells and synthesized TPEs are unable to act as full agonists resembling more anti-estrogens 4-OHT and Endoxifen; C: E2 and all TPEs are able to increase the activity of luciferase in T47D:C4:2 cells transiently transfected with wild-type ER DNA construct; D: E2 is the only agonist in D351G ER mutant T47D:C4:2 cells, as TPEs are unable to increase the luciferase activity in cells expressing the mutant form of ER, indicating the importance of Asp351 of the ER for activation with non-planar TPEs.

of the TPE:ER complex. Based on the X-ray crystallography of the ER in complex with 4OHTAM (Shiau et al. 1998) and raloxifene (Brzozowski et al. 1997), it was determined that the basic side chains of these antiestrogens are in proximity of Asp351 in the ER. It was hypothesized that this interaction with raloxifene actually neutralizes and shields Asp351 preventing it from interacting with ligand-independent activating function 1 (AF-1). In contrast, 4OHTAM possesses some estrogenic activity, because the side chain is too short (Shiau et al. 1998). Substitution of Asp351 with Glycine which is a non-charged aminoacid, leads to loss of estrogenic activity of the ER bound with 4OHTAM (MacGregor Schafer et al. 2000; Levenson et al. 2001). Results from ERE luciferase assays in T47:C4:2 cells transiently transfected with wild type and D351G mutant ER expression plasmids demonstrated that wild type ER was activated by all of the tested TPEs, however substitution of Asp351 by Gly prevented the increase of ERE luciferase activity by all TPEs and only planar E2, which does

not interact with Asp351 at all, or exposes it on the surface of the complex, was able to activate ERE in D351G ER transfected cells. This confirms and expands the classification of estrogens, where planar estrogens such as E2 are classified as class I and all TPE-related estrogens are classified as class II estrogens based on the mechanism of activation of the ER (Jordan et al. 2001).

Further we tested the hypothesis that, the shape of the ER complex with either planar estrogens (Class I) or angular estrogens (Class II), can modulate the apoptotic actions of estrogen through the shape of the resulting complex. In this study MCF-7:5C cells were employed to investigate the actions of 4-OHT and our model TPEs on estradiol-induced apoptosis. As estrogen-induced apoptosis can be reversed in a concentration related manner by the nonsteroidal antiestrogen 4-OHT, paradoxically, all tested TPEs were able to reverse the apoptotic effect of estradiol in MCF-7:5C cells, at the same time the tested TPEs alone were not able to induce apoptosis in these cells significantly (Figure 4B). However, the tested TPEs have still retained their ability to induce ERE-luciferase activity in MCF-7:5C cells, indicating that these compounds are still agonists of the ER in these cells, but biologically acted as antagonists. Besides differences in biological effects of TPEs in MCF-7 cells and MCF-7:5C cells, biochemical effects of tested TPEs on ER complex similar to those with 4-OHT were studied. 4-OHT is known to retard the destruction of the 4-OHT ER complex (Pink and Jordan 1996; Wijayaratne and McDonnell 2001). Similarly, the TPEs do not facilitate the rapid destruction of the TPE:ER complex, as it was shown via Western blotting that the TPE:ER levels are analogous to 4-OHT:ER levels rather than estradiol ER-like, where ER is rapidly degraded. As it was noted previously, ER degradation plays a crucial role in estrogen-mediated gene expression. It was previously shown that ER protein degradation is proteasome mediated (Lonard et al. 2000; Reid et al. 2003), and ER coactivator SRC3/AIB1 links the transcriptional activity of the receptor and its proteasome degradation (Shao et al. 2004). Our results indicate that the transcriptional activity of ER, based on qRT-PCR results, is similar on the pS2 gene in both MCF-7:WS8 cells and MCF-7:5C cells with the tested TPE compounds, and based on our ChIP assay results for evaluating the ER's recruitment on the pS2 gene promoter, the E2:ER complex has robust binding in the promoter region and SRC-3 is detected presumably bound to the ER complex, however, 4-OHT:ER complexes only have modest binding of ER α and virtually no SRC-3 in the promoter region, at the same time, the TPEs permit some binding of the TPE:ER complexes in the promoter region but there are lower levels of SRC-3 and a reduced ability to stimulate PS2 mRNA synthesis (Figure 5).

We believe that the changed conformation of the TPE:ER complex, prevents the complete closure of H12 over the ligand-binding cavity and thus does not allow co-activators to bind to the incompletely open AF-2 motif on the ER's surface. Indeed, LeClerc's group (Bourgoin-Voillard et al. 2010) have recently confirmed and extended our molecular classifications of estrogens, with a larger series of compounds and have also shown that an angular TPE does not cause the destruction of the ER complex in a manner analogous to estradiol when MCF-7 cells are examined by immunohistochemistry for the ER, and that the putative Class II estrogens that do not permit the appropriate sealing of the LBD with helix 12 do not efficiently bind co-activators, therefore our respective studies are in agreement.

In summary, the proposed hypothesis that the TPE-ER complex significantly changes the shape of the ER to adopt a conformation that mimics that adopted by 4-OHT when it binds to the ER. A co-activator now has difficulty in binding to the TPE-ER complex

appropriately, but whereas this does affect cell replication, it dramatically impairs the events that must be triggered to cause apoptosis. Future studies will confirm or refute our hypothesis based upon the known intrinsic activity of mutant ERs and their capacity to investigate estrogen-target genes.

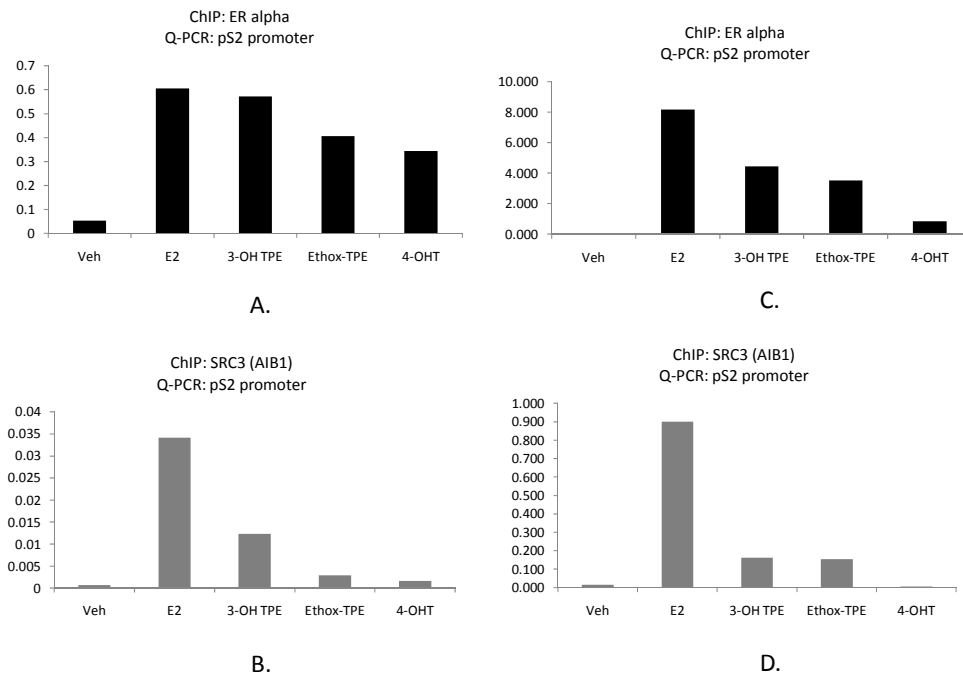


Fig. 5. A&B: ChIP analysis performed in MCF-7:WS8 cells with pS2 promoter region was pulled down via anti-ERα antibody (A) and anti-SRC3/AIB1 antibody (B); C&D: ChIP analysis performed in MCF-7:5C cells with pS2 promoter region pulled down via anti-ERα antibody (C) and anti-SRC3/AIB1 antibody (D). All results indicate that in both cell lines tested TPEs and E2 recruit ERα complex to the pS2 promoter region, but interestingly, class II estrogens are unable to co-recruit sufficient amount of SRC-3 co-activator, unlike E2.

6. Relevance to current clinical research

Laboratory studies show that low concentrations of estrogen can cause apoptotic death of breast tumor cells, following estrogen deprivation with antihormonal treatment. This has translated very well into the clinic, and recent clinical trials have demonstrated that low-dose estrogen treatment can effectively be utilized after the formation of resistance to antihormonal treatment. Ellis and colleagues (Ellis et al. 2009) have shown, that a daily dose of 6 mg of estradiol could stop the growth of tumors or even cause them to shrink in about 25% of women with metastatic breast cancer that had developed resistance to antihormonal therapy. At the same time, these results correlate with earlier results obtained by Loenning and coworkers (Lonning et al. 2001), who have studied the efficacy of high dose of DES on the responsiveness of metastatic breast cancer following exhaustive antihormonal treatment

with tamoxifen, aromatase inhibitors and etc. 4 out of 32 patients had complete responses (Lonning et al. 2001) and 1 patient after 5 year treatment with DES had no recurrence for a following 6 years (Lonning 2009). The question at that moment remains whether estrogen at physiologic concentrations can be efficient as antitumor agent in estrogen-deprived breast tumors. As mentioned previously, Ellis and coworkers have demonstrated that an equivalent clinical benefit for high (30 mg daily) and low (6 mg daily) dose of estradiol in metastatic breast cancer patients who had failed aromatase inhibitor therapy, which is long-term estrogen deprivation. Overall, the results demonstrate that low dose estrogen therapy has fewer systemic sideeffects, but the same efficacy as a treatment for long-term antihormone resistant breast cancer as high dose estrogen therapy. This can be seen as "replacement with" physiologic estrogen to premenopausal levels. The benefit-risk ratio is in favor of low-dose estrogen therapy. These results correlate well with results from WHI trial of estrogen-replacement therapy (ERT) in hysterectomized postmenopausal women (LaCroix et al. 2011). The WHI results show a sustained reduction in the incidence of breast cancer in postmenopausal women up to 5 years after the intervention with conjugated equine estrogens for 5 years prior. It was demonstrated that the group of patients receiving conjugated equine estrogens had incidence of breast cancer 0.27% in comparison to the control group of patients the incidence was 0.35%. The idea that woman's own estrogen can act as an antitumor agent after estrogen-deprivation to prevent metastization and tumor growth (Wolf and Jordan 1993) has lead to incorporation into the Study of Letrozole Extension (SOLE) trial. This trial is addressing the question whether regular drug holidays can decrease recurrence of breast cancer by physiologic estrogen after deprivation with aromatase inhibitor letrozole. Subsequent trials may have to use ERT for a few weeks to trigger apoptosis.

7. Conclusion

Taken together, the demonstrations of the apoptotic actions of estrogen as a potential anticancer agent in postmenopausal breast cancer patients, now provides a rationale to further explore and decipher mechanisms of estrogen-induced apoptosis. There is a possibility that future studies on the molecular mechanism of estrogen-induced apoptosis will help to identify new more safer and specific agents for breast cancer therapy.

8. Acknowledgments

This work was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence (principal investigator V. Craig Jordan); subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen For The Cure Foundation under Award number SAC100009 (international postdoctoral fellow Philipp Y. Maximov) and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

9. References

Allen, E., Doisy, E.A. (1923). An ovarian hormone: Preliminary reports on its localization extraction and partial purification and action in test animals. *JAMA* 81: 810-821.

Anderson, C. P., J. M. Tsai, et al. (1999). Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cell lines via apoptosis. *Exp Cell Res* 246(1): 183-192.

Balaburski, G. M., R. C. Dardes, et al. (2010). Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 37(2): 387-398.

Beatson, G. T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* 2: 162-165.

Bourgoin-Voillard, S., D. Gallo, et al. (2010). Capacity of type I and II ligands to confer to estrogen receptor alpha an appropriate conformation for the recruitment of coactivators containing a LxxLL motif-Relationship with the regulation of receptor level and ERE-dependent transcription in MCF-7 cells. *Biochem Pharmacol* 79(5): 746-757.

Boyd, S. (1900). On Oophorectomy in cancer of the breast. *British Medical Journal* 2: 134-141.

Brodie, A. H., D. Jelovac, et al. (2003). The intratumoral aromatase model: studies with aromatase inhibitors and antiestrogens. *J Steroid Biochem Mol Biol* 86(3-5): 283-288.

Brzozowski, A. M., A. C. Pike, et al. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389(6652): 753-758.

Cummings, S. R., S. Eckert, et al. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 281(23): 2189-2197.

Dodds, E. C., Goldberg, L., Lawson, W., Robinson, R. (1938). Estrogenic activity of certain synthetic compounds. *Nature* 141: 247-248.

EBCTCG (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 365(9472): 1687-1717.

Ellis, M. J., F. Gao, et al. (2009). Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* 302(7): 774-780.

Fisher, B., J. P. Costantino, et al. (1998). Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 90(18): 1371-1388.

Fisher, B., J. Dignam, et al. (1999). Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomised controlled trial. *Lancet* 353(9169): 1993-2000.

Folca, P. J., R. F. Glascock, et al. (1961). Studies with tritium-labelled hexoestrol in advanced breast cancer. Comparison of tissue accumulation of hexoestrol with response to bilateral adrenalectomy and oophorectomy. *Lancet* 2(7206): 796-798.

Gail, M. H., J. P. Costantino, et al. (1999). Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst* 91(21): 1829-1846.

Glascock, R. F. and W. G. Hoekstra (1959). Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *Biochem J* 72: 673-682.

- Goss, P. E., J. N. Ingle, et al. (2005). Randomized trial of letrozole following tamoxifen as extended adjuvant therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *J Natl Cancer Inst* 97(17): 1262-1271.
- Gottardis, M. M. and V. C. Jordan (1987). Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 47(15): 4020-4024.
- Gottardis, M. M. and V. C. Jordan (1988). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 48(18): 5183-5187.
- Gottardis, M. M., M. E. Ricchio, et al. (1990). Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res* 50(11): 3189-3192.
- Gottardis, M. M., S. P. Robinson, et al. (1988). Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 48(4): 812-815.
- Gust, R., R. Keilitz, et al. (2001). Investigations of new lead structures for the design of selective estrogen receptor modulators. *J Med Chem* 44(12): 1963-1970.
- Haddow, A. (1950). The chemotherapy of cancer. *Br Med J* 2(4691): 1271-1272.
- Haddow, A. (1970). David A. Karnofsky memorial lecture. Thoughts on chemical therapy. *Cancer* 26(4): 737-754.
- Haddow, A., Watkinson, J.M., Paterson, E. (1944). Influence of synthetic oestrogens upon advanced malignant disease. *British Medical Journal*: 393-398.
- Harper, M. J. and A. L. Walpole (1967). A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil* 13(1): 101-119.
- Herman, M. E. and B. S. Katzenellenbogen (1996). Response-specific antiestrogen resistance in a newly characterized MCF-7 human breast cancer cell line resulting from long-term exposure to trans-hydroxytamoxifen. *J Steroid Biochem Mol Biol* 59(2): 121-134.
- Howell, A., J. Cuzick, et al. (2005). Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet* 365(9453): 60-62.
- Howell, A., D. J. Dodwell, et al. (1992). Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* 3(8): 611-617.
- Ingle, J. N., D. L. Ahmann, et al. (1981). Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 304(1): 16-21.
- Jensen, E. V., Jacobson, H.I. (1962). Basic guidelines to the mechanism of estrogen action. *Recent Progr. Hormone Res.* 18: 387-414.
- Jensen, E. V. and V. C. Jordan (2003). The estrogen receptor: a model for molecular medicine. *Clin Cancer Res* 9(6): 1980-1989.
- Jordan, V. C. (2001). Selective estrogen receptor modulation: a personal perspective. *Cancer Res* 61(15): 5683-5687.
- Jordan, V. C. (2003). Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2(3): 205-213.
- Jordan, V. C. (2004). Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 5(3): 207-213.

- Jordan, V. C. (2007). SERMs: meeting the promise of multifunctional medicines. *J Natl Cancer Inst* 99(5): 350-356.
- Jordan, V. C. (2008). Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 44(1): 30-38.
- Jordan, V. C. and A. M. Brodie (2007). Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 72(1): 7-25.
- Jordan, V. C. and L. G. Ford (2011). Paradoxical Clinical Effect of Estrogen on Breast Cancer Risk: A "New" Biology of Estrogen-induced Apoptosis. *Cancer Prev Res (Phila)* 4(5): 633-637.
- Jordan, V. C., R. Koch, et al. (1986). Oestrogenic and antioestrogenic actions in a series of triphenylbut-1-enes: modulation of prolactin synthesis in vitro. *Br J Pharmacol* 87(1): 217-223.
- Jordan, V. C., M. E. Lieberman, et al. (1984). Structural requirements for the pharmacological activity of nonsteroidal antiestrogens in vitro. *Mol Pharmacol* 26(2): 272-278.
- Jordan, V. C., E. Phelps, et al. (1987). Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 10(1): 31-35.
- Jordan, V. C. and S. P. Robinson (1987). Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 46(5): 1870-1874.
- Jordan, V. C., J. M. Schafer, et al. (2001). Molecular classification of estrogens. *Cancer Res* 61(18): 6619-6623.
- Kennedy, B. J. (1965). Hormone therapy for advanced breast cancer. *Cancer* 18(12): 1551-1557.
- Kennedy, B. J. (1965). Systemic effects of androgenic and estrogenic hormones in advanced breast cancer. *J Am Geriatr Soc* 13: 230-235.
- Kennedy, B. J. and I. T. Nathanson (1953). Effects of intensive sex steroid hormone therapy in advanced breast cancer. *J Am Med Assoc* 152(12): 1135-1141.
- Lacassagne, A. (1936). Hormonal pathogenesis of adenocarcinoma of the breast. *Am J Cancer* 27: 217-225.
- LaCroix, A. Z., R. T. Chlebowski, et al. (2011). Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 305(13): 1305-1314.
- Lathrop, A. E. C., Loeb, L. (1916). Further Investigations on the Origin of Tumors in Mice. *J Cancer Res* 1(1): 1-19.
- Lee, E. S., J. M. Schafer, et al. (2000). Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6(12): 4893-4899.
- Lerner, L. J., F. J. Holthaus, Jr., et al. (1958). A non-steroidal estrogen antiagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenylethanol. *Endocrinology* 63(3): 295-318.
- Lerner, L. J. and V. C. Jordan (1990). Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 50(14): 4177-4189.
- Levenson, A. S., W. H. Catherino, et al. (1997). Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. *J Steroid Biochem Mol Biol* 60(5-6): 261-268.

- Levenson, A. S. and V. C. Jordan (1998). The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 58(9): 1872-1875.
- Levenson, A. S., J. I. MacGregor Schafer, et al. (2001). Control of the estrogen-like actions of the tamoxifen-estrogen receptor complex by the surface amino acid at position 351. *J Steroid Biochem Mol Biol* 76(1-5): 61-70.
- Levenson, A. S., D. A. Tonetti, et al. (1998). The oestrogen-like effect of 4-hydroxytamoxifen on induction of transforming growth factor alpha mRNA in MDA-MB-231 breast cancer cells stably expressing the oestrogen receptor. *Br J Cancer* 77(11): 1812-1819.
- Lewis-Wambi, J. S., H. R. Kim, et al. (2008). Buthionine sulfoximine sensitizes antihormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res* 10(6): R104.
- Lewis, J. S., K. Meeke, et al. (2005). Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 97(23): 1746-1759.
- Lewis, J. S., C. Osipo, et al. (2005). Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 94(1-3): 131-141.
- Lieberman, M. E., J. Gorski, et al. (1983). An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* 258(8): 4741-4745.
- Liu, H., E. S. Lee, et al. (2003). Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 95(21): 1586-1597.
- Lonard, D. M., Z. Nawaz, et al. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol Cell* 5(6): 939-948.
- Lonning, P. E. (2009). Additive endocrine therapy for advanced breast cancer - back to the future. *Acta Oncol* 48(8): 1092-1101.
- Lonning, P. E., P. D. Taylor, et al. (2001). High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 67(2): 111-116.
- MacGregor Schafer, J., H. Liu, et al. (2000). Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* 60(18): 5097-5105.
- Maximov, P. Y., C. B. Myers, et al. (2010). Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen. *J Med Chem* 53(8): 3273-3283.
- O'Regan, R. M., C. Gajdos, et al. (2002). Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *J Natl Cancer Inst* 94(4): 274-283.
- Osborne, C. K., V. Bardou, et al. (2003). Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 95(5): 353-361.
- Osborne, C. K., E. Coronado, et al. (1991). Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* 83(20): 1477-1482.

- Osipo, C., C. Gajdos, et al. (2003). Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 95(21): 1597-1608.
- Park, W. C., H. Liu, et al. (2005). Deregulation of estrogen induced telomerase activity in tamoxifen-resistant breast cancer cells. *Int J Oncol* 27(5): 1459-1466.
- Pink, J. J., M. M. Bilimoria, et al. (1996). Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* 74(8): 1227-1236.
- Pink, J. J. and V. C. Jordan (1996). Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 56(10): 2321-2330.
- Reid, G., M. R. Hubner, et al. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* 11(3): 695-707.
- Robinson, S. P. and V. C. Jordan (1989). Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. *Cancer Res* 49(7): 1758-1762.
- Robson, J. M., Schonberg, A. (1937). Estrous reactions, including mating, produced by triphenyl ethylene. *Nature* 140: 196.
- Schroder, C. P., A. K. Godwin, et al. (1996). Glutathione and drug resistance. *Cancer Invest* 14(2): 158-168.
- Shao, W., E. K. Keeton, et al. (2004). Coactivator AIB1 links estrogen receptor transcriptional activity and stability. *Proc Natl Acad Sci U S A* 101(32): 11599-11604.
- Shiau, A. K., D. Barstad, et al. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95(7): 927-937.
- Song, R. X., G. Mor, et al. (2001). Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. *J Natl Cancer Inst* 93(22): 1714-1723.
- Toft, D. and J. Gorski (1966). A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A* 55(6): 1574-1581.
- Townsend, D. M., K. D. Tew, et al. (2003). The importance of glutathione in human disease. *Biomed Pharmacother* 57(3-4): 145-155.
- Voehringer, D. W. (1999). BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic Biol Med* 27(9-10): 945-950.
- Vogel, V. G., J. P. Costantino, et al. (2006). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 295(23): 2727-2741.
- Vogel, V. G., J. P. Costantino, et al. (2010). Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)* 3(6): 696-706.
- Walpole, A. L., Patterson, E. (1948). Synthetic oestrogens in mammary cancer. *Lancet* 2(2583): 783-786.
- Wijayaratne, A. L. and D. P. McDonnell (2001). The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol Chem* 276(38): 35684-35692.

- Wolf, D. M. and V. C. Jordan (1993). A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 127: 23-33.
- Yao, K., E. S. Lee, et al. (2000). Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 6(5): 2028-2036.

Models and mechanisms of acquired antihormone resistance in breast cancer: significant clinical progress despite limitations¹⁾

Elizabeth E. Sweeney, Russell E. McDaniel,
Philipp Y. Maximov, Ping Fan and V. Craig Jordan*

Department of Oncology, Lombardi Comprehensive
Cancer Center, Georgetown University Medical Center,
Washington, DC, USA

Abstract

Translational research for the treatment and prevention of breast cancer depends upon the four Ms: models, molecules, and mechanisms in order to create medicines. The process, to target the estrogen receptor (ER) in estrogen-dependent breast cancer, has yielded significant advances in patient survivorship and the first approved medicines (tamoxifen and raloxifene) to reduce the incidence of any cancer in high- or low-risk women. This review focuses on the critical role of the few ER-positive cell lines (MCF-7, T47D, BT474, ZR-75-1) that continue to advance our understanding of the estrogen-regulated biology of breast cancer. More importantly, the model cell lines have provided an opportunity to document the development and evolution of acquired antihormone resistance. The description of this evolutionary process that occurs in micrometastatic disease during up to a decade of adjuvant therapy would not be possible in the patient. The use of the MCF-7 breast cancer cell line, in particular, has been instrumental in discovering a vulnerability of ER-positive breast cancer exhaustively treated with antihormone therapy. Physiologic estradiol acts as an apoptotic trigger to cause tumor regression. These unanticipated findings in the laboratory have translated to clinical advances in our knowledge of the paradoxical role of estrogen in the life and death of breast cancer.

Keywords: antihormone; breast cancer; resistance.

¹⁾Never in the field of breast cancer research [human conflict] was so much owed by so many to so few.

(With apologies to the late Winston Spencer Churchill, Prime Minister, August 20, 1940 reporting on the successful winning of the Battle of Britain.)

*Corresponding author: V. Craig Jordan, Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Rd, NW Research Building, Suite E501 Washington, DC 20057, USA
Phone: +202 687 2897, Fax: +202 687 6402,
E-mail: vcj2@georgetown.edu

Received December 16, 2011; accepted January 12, 2012;
previously published online February 20, 2012

Introduction

The past four decades have witnessed the successful evolution of effective breast cancer therapies as scientific research has translated into clinical practice. Breast cancer therapy began its story with combination cytotoxic chemotherapy. Chemotherapy, though able to create complete responses in some cases of breast cancer, works non-specifically, causing harmful and sometimes intolerable, life-threatening side effects. Antiestrogen therapies, by contrast, provide significant therapeutic improvement by focusing on a target, the tumor estrogen receptor (ER) [1]. It is important to point out that the ER was initially used not as a therapeutic target, but as a predictor of response to endocrine ablation, such as oophorectomy [2]. The innovation of targeting the tumor ER specifically using the non-steroidal antiestrogen tamoxifen (Figure 1) [3] ultimately changed the prognosis of women with breast cancer by proposing two new treatment strategies: a new approach to therapy with long-term early adjuvant tamoxifen treatment following surgery and, subsequently, the possibility of using tamoxifen for chemoprevention [1, 2]. In both cases, the target would be the ER, to be blocked by tamoxifen.

Tamoxifen is approved by the Food and Drug Administration (FDA) to treat node-positive and node-negative breast cancer patients with long-term adjuvant therapy and is approved to lower the incidence of breast cancer in high-risk pre- and postmenopausal women. In both applications, clinical trials established and confirmed that patients with ER-positive breast cancer are the ones who benefit. Tumors that are ER-negative do not respond to tamoxifen. In addition to blocking estrogen's binding to its receptor, another means of limiting estrogenic activity in breast tissue is by blocking the synthesis of estrogen. Aromatase inhibitors block estrogen's conversion from its androgen precursor thereby limiting the production of estrogen [4, 5]. This approach has proven beneficial clinically with fewer side effects than tamoxifen and improvements in recurrence rates and survival for postmenopausal patients [6–9].

The benefit of antihormone (data primarily from tamoxifen trials) therapy targeted to the ER is impressive in terms of both recurrence-free survival and decreases in mortality [7, 10]. Millions of women now live longer, healthier lives based on the application of translational research [1]. Women of any age with ER-positive tumors experience an approximately 30% mortality reduction when treated with long-term (5 year) adjuvant tamoxifen [7, 10]. Postmenopausal women, however, receive greater clinical benefit with aromatase

inhibitors rather than tamoxifen, in terms of lower breast cancer recurrence rates and fewer side effects [6]. Aromatase inhibitors can be used instead of tamoxifen for 5 years, after tamoxifen for 5 years, or by switching to an aromatase inhibitor after a year or two of tamoxifen. The important principle is to ensure compliance so that at least 5 years of antihormone treatment is used.

Breast cancer prevention trials built on the previous clinical experience with tamoxifen to demonstrate tamoxifen's efficacy in preventing ER-positive invasive breast cancer in women at high-risk [11]. However, few high-risk women benefit from population-based chemoprevention with tamoxifen, while many are exposed to side effects such as endometrial cancer and thromboembolic events [12]. As a result, a paradigm shift occurred with the finding that nonsteroidal antiestrogens are, in fact, selective ER modulators (SERMs). The laboratory discovery that SERMs can maintain bone density but prevent mammary carcinogenesis led to the idea of treating osteoporosis while preventing breast cancer at the same time [13–15]. It is fair to say that the laboratory finding [16] that tamoxifen increases the growth of human endometrial cancer but stops the growth of breast cancer, and its subsequent clinical confirmation [16, 17] really stressed the need to find a new chemopreventive medicine. Raloxifene is a drug similar in structure to tamoxifen (Figure 1), which is now prescribed indefinitely as a medicine to prevent osteoporosis, offering a beneficial side effect of breast cancer prevention in postmenopausal women [18, 19]. Additionally, raloxifene

is FDA-approved as a prevention strategy to reduce the incidence of ER-positive breast cancer in at-risk postmenopausal women without increasing the incidence of endometrial cancer as occurs with tamoxifen [20, 21]. Figure 1 illustrates the structures of estradiol, raloxifene, tamoxifen, and related metabolites.

With this brief clinical background of progress in the quality of life and survivorship for women with breast cancer, and the practical progress in reducing the incidence of breast cancer, several principles emerge to focus laboratory efforts to enhance further advances. Long-term therapy is the key to successful increases in survivorship, and only ER-positive tumors are responsive to antihormone therapy. However, because of the finding that 5 or more years of therapy can control recurrences of the growth of micrometastatic primary breast cancer, it is acquired resistance to antihormone therapy that must be addressed. Models must replicate clinical experience with the ER-positive tumor. The surviving cells whose growth is not blocked by antihormones have the plasticity to respond to treatment in a Darwinian model of continued growth and replication.

We will first describe the limited types of ER-positive breast cancer cells available to the scientific community and the strategies used in the laboratory to create models to mimic clinical experience, i.e., years of antihormone therapy. Through the creation of reproducible models, mechanisms can be deciphered to apply to new clinical treatment strategies.

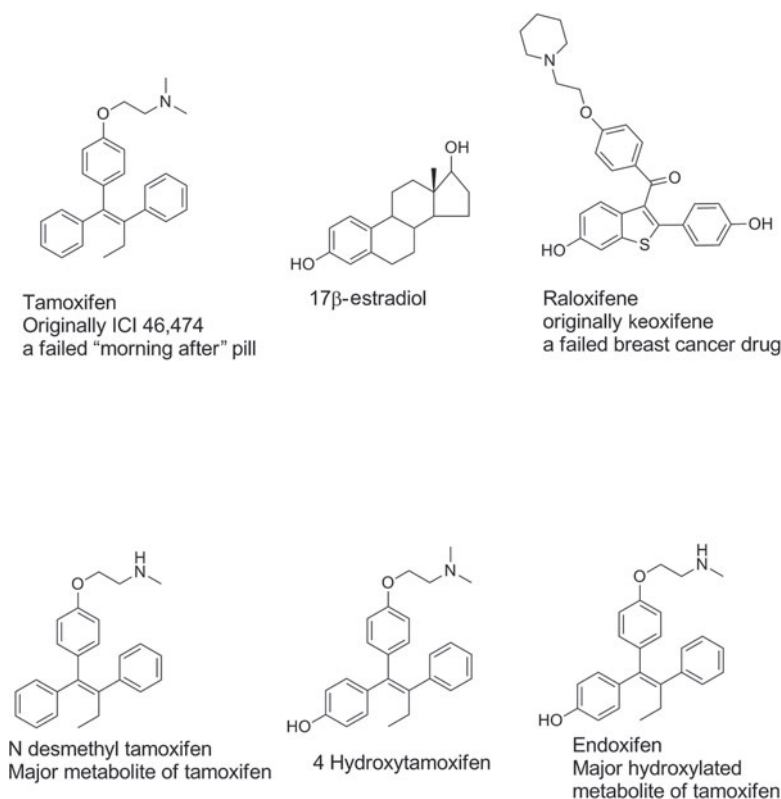


Figure 1 Chemical structures of 17β-estradiol, raloxifene, tamoxifen, and tamoxifen's metabolites n-desmethyl tamoxifen, 4-hydroxytamoxifen, and endoxifen.

Cell lines as platforms for modeling acquired antihormone resistance

The Early Breast Cancer Trialists' Collaborative Group recently showed that after about 5 years of tamoxifen therapy for women with ER-positive breast tumors (10,645 women), yearly breast cancer mortality rate was reduced by 30% for 15 years after treatment initiation [7]. If we estimate that ER-positive breast cancer, the most prevalent type, accounts for 75% of all breast cancer, it follows that about half of the breast cancers may have or acquire resistance to antihormone therapy. This, combined with the fact that over 200,000 new cases of breast cancer [22] are expected to occur each year, makes acquired resistance a critical issue in breast cancer research and women's health. Prevention of primary breast cancer or the maintenance of patients to prevent recurrence of the disease is an important advance in translational research that continues to reduce healthcare costs and improve survivorship for millions of patients worldwide. Although it is fair to say that few women at high risk for breast cancer elect the chemoprevention option, there are more than half a million women using raloxifene to prevent osteoporosis and prevent breast cancer at the same time [18]. However, tumors that form during long-term raloxifene treatment [19] have acquired resistance to this SERM.

It is currently impossible to analyze the cell biology of every patient's individual breast tumor and predict outcomes, both practically and financially. The actual relationship of the cancer cell with supporting stroma of an individual tumor cannot yet be reconstructed under laboratory conditions, but what can be achieved at this stage is the interrogation of available cell lines to focus on a specific group of ER-positive tumors and obtain general principles with which to plan treatments.

In other words, laboratory models in vitro and in vivo represent the medium for a conversation between the laboratory and the clinic. These models represent important subgroups of breast tumors in patients.

Breast cancer cell lines that are ER-positive are of specific value to conduct translational research to understand the mechanisms by which hormone-responsive breast tumors may develop acquired antihormone resistance. The ER-positive models to be discussed here are: ZR-75-1, BT-474, T47D, and MCF-7. Each cell line is available from the American Type Culture Collection (ATCC), but there are individual variants maintained in specific laboratories. The current ER statuses (Figure 2), ER protein regulation (Figure 2), hormone responsiveness to the principal steroidal estrogens estradiol and estrone (Figure 3), and the relative ability of tamoxifen and its metabolites to block combined circulating levels of estrone and estradiol (Figure 4) are illustrated. All cells tested have been confirmed by DNA fingerprinting.

The ZR-75-1 breast cancer cell line

The ZR-75-1 human breast cancer cell line was derived in the late 1970s from a 63-year-old postmenopausal female patient with metastatic ductal carcinoma of the breast. The cells were taken from the ascites 3 months after initiation of tamoxifen treatment and exhibit estrogen and insulin responsiveness [25]. As ZR-75-1 cells are passaged, they retain their epithelial morphology, remaining similar in appearance to their original source biopsy, though their chromosome count decreases from approximately 75 to 72 after 38 passages [25]. ZR-75-1 cells are ER-positive, glucocorticoid receptor (GR)-positive, androgen receptor (AR)-positive, and progesterone receptor

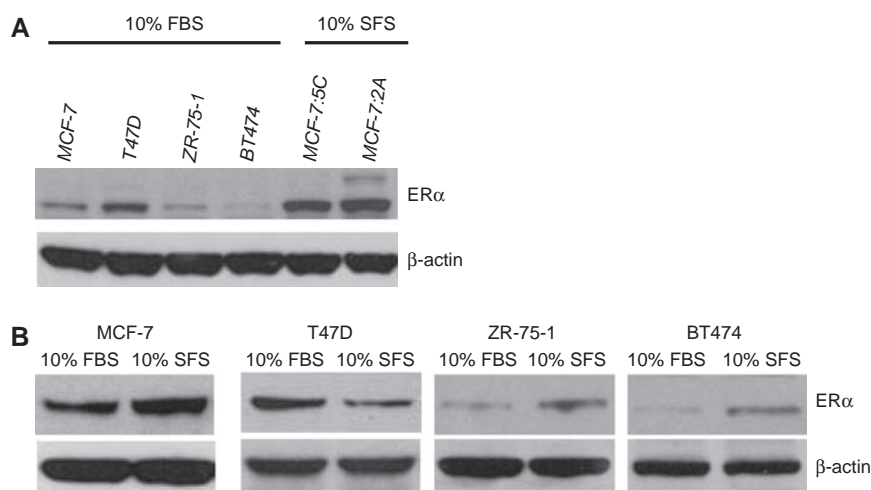


Figure 2 (A) ERα expression levels in different ER-positive cells. Cell lysates of MCF-7, T47D, ZR-75-1, BT474, MCF-7:5C, and MCF-7:2A were harvested. MCF-7, T47D, ZR-75-1, and BT474 cells were cultured under conditions with estrogen (10% FBS), while MCF-7:5C and MCF-7:2A cells were cultured under estrogen-free conditions (10% SFS). ERα expression levels were examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control. (B) Modulation of ERα expression in the absence of estrogen. Wild-type ER-positive MCF-7, T47D, ZR-75-1, and BT474 cells were cultured under conditions with estrogen (10% FBS) or without estrogen (10% SFS) for 3 days, respectively. Cell lysates were harvested. ERα expression levels were examined by immunoblotting with primary antibody. Immunoblotting for β-actin was determined for loading control.

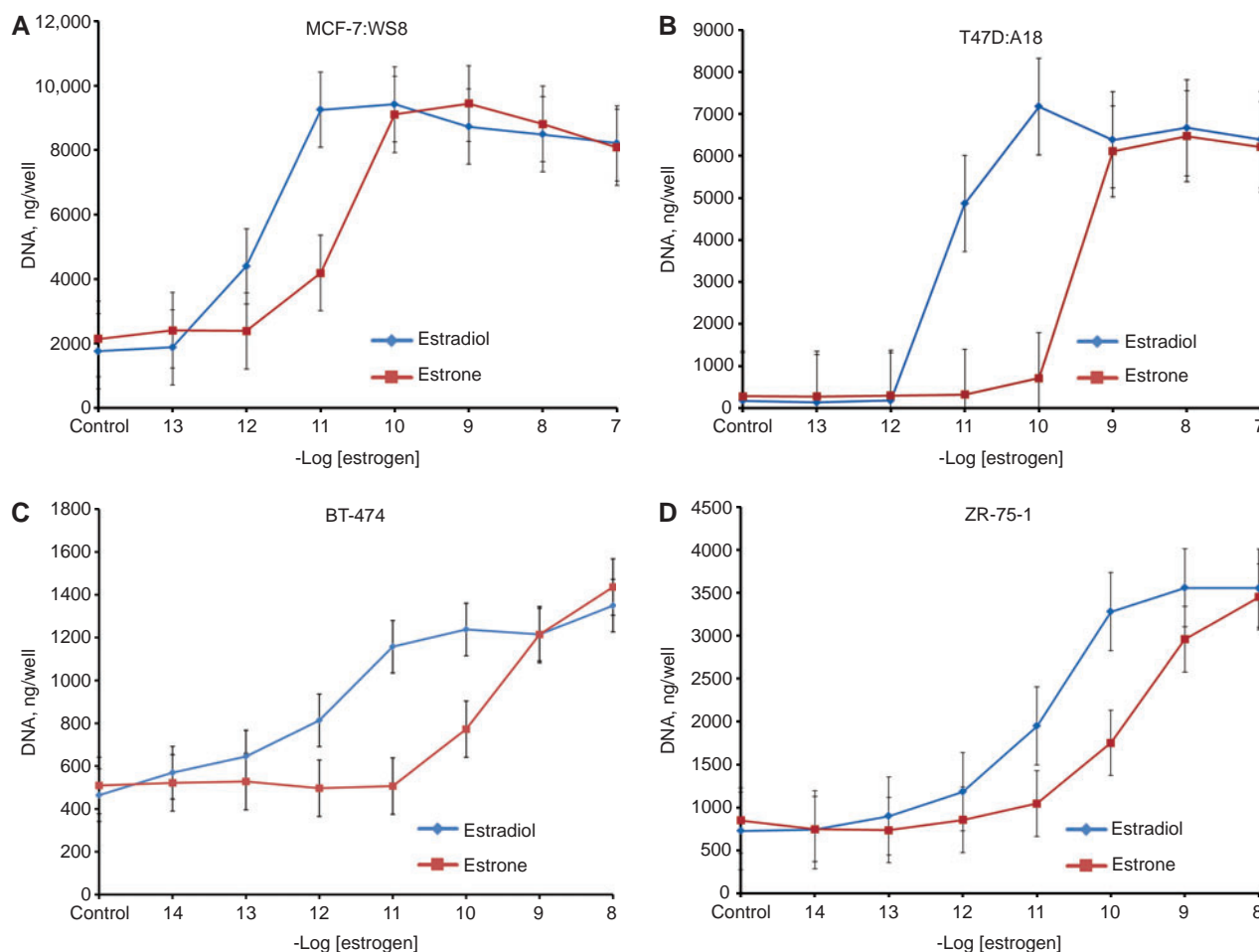


Figure 3 Proliferative responses of different ER-positive breast cancer cell lines to treatments with estradiol (E_2) and estrone (E_1). Growth of cells was determined by measuring DNA per well after 7 day treatments. (A) MCF-7:WS8 cells, hypersensitive clones of MCF-7 cell line; (B) T47D:A18 cells, hypersensitive clone of T47D cell line; (C) BT-474 ER-positive breast cancer cells (ATCC); (D) ZR-75-1 ER-positive breast cancer cells (ATCC). Estradiol is the most potent of the natural estrogens in a woman's body and estrone, with the 17β hydroxyl oxidized to a ketone, is less potent. It does, however, significantly contribute to breast cancer cell growth.

(PR)-positive [25]. Tamoxifen (10^{-6} M) causes growth inhibition and the cells die [26]. Also, the cells are specifically growth stimulated by insulin and inhibited by androgens and glucocorticoids [25].

The BT-474 breast cancer cell line

The BT-474 cell line comprises ER-positive, PR-positive epithelial cancer cells derived from invasive ductal breast carcinoma of a 60-year-old female patient [27]. Notably, these cells also express the membrane receptor human epidermal growth factor receptor 2 (HER2) [28]. With 55 chromosomes, they grow in adherent patches in tissue culture and are tumorigenic [27]. BT-474 cells grow in response to estradiol, via their ER (see Figure 3).

The T47D breast cancer cell line

The T47D cell line originates from a pleural effusion of a 54-year-old female patient with infiltrating ductal breast

carcinoma. The cells have approximately 60 to 70 chromosomes, multiple mitochondria, and irregular nuclei and nucleoli [29]. They maintain their epithelial morphology after several years of passage, can produce casein, and can be grown in a monolayer in vitro [29]. First described as an ER-positive, PR-positive, AR-positive, GR-positive, epithelial cell carcinoma model, it has since been established that the nuclear receptor levels and hormone responsiveness depend on the culture conditions [30]. T47D cells express ER and PR in estrogen-rich media but lose most PR and ER expression when grown in the absence of estrogen [30].

Classically, estradiol stimulates proliferation of the T47D cell line through the ER and stimulates estrogen-regulated proteins such as PR, while tamoxifen inhibits this growth [31]. The stimulatory action of physiologic estrogens and the inhibition caused by tamoxifen and its principal metabolites are shown in Figures 3 and 4, respectively. Without the nuclear receptors, however, neither estradiol nor tamoxifen can influence growth as their mechanism of action through ER is eliminated [30].

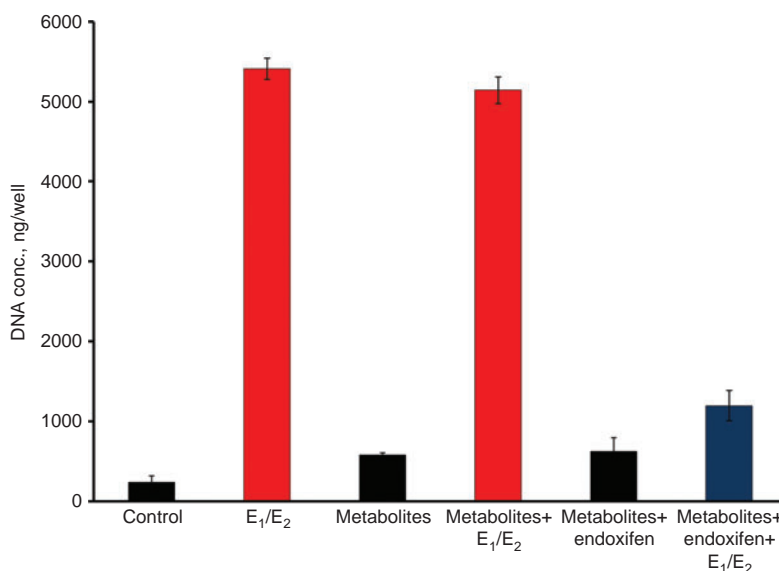


Figure 4 Biological response of MCF-7 cells after 7-day treatment with premenopausal levels of estrone (E₁, 8 nM) and estradiol (E₂, 4 nM) found in plasma of premenopausal women during follicular phase of menstrual cycle [23] and tamoxifen metabolites 4OHT (6.3 nM), N-desmethyl-Tam (558 nM), tamoxifen (386 nM), and endoxifen (35.6 nM) at concentrations found in plasma of extensive metabolizers of tamoxifen [24]. As shown in the figure, a combination of E₁/E₂ induces cell growth, and treatment with a combination of tamoxifen and its metabolites has minor effect on cells. Combination treatment of E₁/E₂ and tamoxifen metabolites does not ablate the proliferation of the cells. However, addition of another tamoxifen metabolite endoxifen at concentrations found in plasma of extensive metabolizers of tamoxifen (35.6 nM) produces almost complete inhibitory effect on cell growth. Treatment with combination of all tamoxifen metabolites (including endoxifen) does not have any major biological effect. Similar results occur with T47D.

The MCF-7 breast cancer cell line

The majority of investigations into acquired antiestrogen drug resistance have utilized the MCF-7 cell line so prevalent in breast cancer laboratories. The MCF-7 cell line has been the topic of an earlier review [32]. MCF-7 cells are used ubiquitously in research for ER-positive breast cancer cell experiments, and many subclones have been established, representing different classes of ER-positive tumors with varying nuclear receptor expression levels.

The MCF-7 cell line was derived from the pleural effusion of a 69-year-old female patient with a diagnosis of adenocarcinoma of the breast [33]. This particular patient had undergone 3 years of radiotherapy and hormone therapy, most likely high-dose diethylstilbestrol (DES), a synthetic estrogen (the cell line was created before tamoxifen was available for clinical use). The cells were noted to be ER-positive [34]. In the mid-1970s, Lippman et al. [35, 36] demonstrated that non-steroidal antiestrogens, in general, and tamoxifen, in particular, could stop the growth of MCF-7 cells in culture, and this could be reversed with the administration of exogenous estradiol.

In the early 1980s, MCF-7 cells were shown to form tumors in vivo [37] with estrogen administration, but estrogen did not significantly stimulate growth of the same cells in vitro [38]. At the time, it was proposed that a factor existing in the animal, but not in culture, be it a second messenger system or peptide growth factor, was required for the profound growth influence of estrogen on MCF-7 cells [38]. However, a landmark discovery occurred in 1986 identifying a contaminant of

phenol red (phenolsulfonphthalein) (Figure 5), the pH indicator in media, as estrogenic [40, 41]. The media was therefore causing cells to grow [41]. All previous studies measuring estrogen's impact on the cells were undermined as the effects were confounded by additional estrogen in the media. The discovery allowed complete withdrawal of estrogen from the cells and the subsequent ability to document the real impact of estrogen on various cell functions including proliferation and apoptosis of MCF-7 cells [42–46].

Being ER-positive, the MCF-7 cell line grows and proliferates with estrogens, in concentrations as low as 10⁻¹¹ M estradiol (Figure 3) [32]. Tamoxifen competitively inhibits

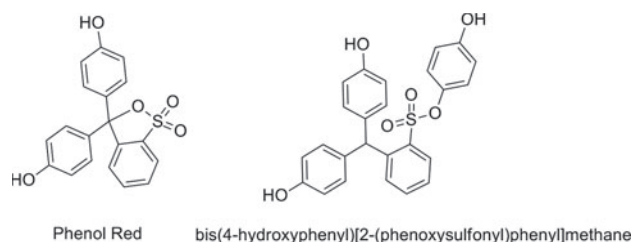


Figure 5 Phenolsulfonphthalein (phenol red), the pH indicator in cell culture media, is structurally similar to the natural estrogen estradiol (Figure 1) and synthetic estrogens. Unlike normal chemical titration analyses that use a pH indicator at very low concentrations, phenol red is incorporated at μM levels in culture media. The estrogenicity was found to vary from batch to batch [39]. However, a potent estrogenic contaminant (right) exerts growth stimulatory effects on breast cancer cells [40].

DNA synthesis in MCF-7 cells, binding to the same ER as do estrogens, though with a 1000-fold lower affinity than estradiol [32]. When added to the cells simultaneously, estradiol can reverse this inhibition at a concentration 100-fold lower than tamoxifen (10^{-7} M vs. 10^{-8} M) causing cell growth (Figure 4) [32]. The actions of tamoxifen and its metabolites on estrogen-stimulated proliferation are shown in Figure 4. Pure antiestrogens, such as fulvestrant, that destroy ER, also inhibit growth of MCF-7 cells [47].

ER regulation in ER-positive breast cancer cell lines

Figure 2 illustrates ER expression in the four described ER-positive breast cancer cell lines in different media conditions. ZR-75-1, BT-474, and MCF-7 cells increase expression of ER in the absence of estrogens, represented here by phenol red-free media supplemented with charcoal-stripped fetal bovine serum (SFS). Estrogen exposure to these cells causes decreased ER mRNA and protein levels [48]. T47D cells, by contrast, express more ER in an estrogenic environment, shown here as red media with fetal bovine serum (FBS) [48]. As previously stated, T47D ER expression is lost in an estrogen-free environment. Tamoxifen causes increased ER protein levels in MCF-7 and T47D cells, while fulvestrant causes decreased protein levels in both cell lines [48]. The alternate models of ER regulation in the cell lines has previously been summarized [48] and is now updated and illustrated in Figure 6 for convenience. The consistent model (Model I) of ER regulation is an upregulation of ER in the absence of estrogen. However, T47D does not conform and requires estrogen for ER synthesis (Model II).

Models of acquired antihormone resistance in vitro

ER-negative breast cancer cells, such as the MDA-MB-231 and SKBr3 cell lines, do not respond to antihormone treatment. There are some ER-positive cell lines that also exhibit intrinsic resistance; that is, antihormones do not create a subpopulation of these cells that are resistant over time. They simply do not respond initially, perhaps via growth factor receptor overexpression allowing other mechanisms of growth stimulation. Osborne's group showed in 1992 [49] that when ER-positive MCF-7 cells are transfected with HER2, the cells are intrinsically resistant to antihormones such as tamoxifen, presenting HER2 as a potentially important factor for tamoxifen sensitivity and drug resistance.

To investigate the properties of acquired antihormone-resistant breast cancer cells, populations of MCF-7 cells have been created that are adapted to various antihormone environments. MCF-7 cells, more than the other three ER-positive cell lines T47D, BT-474, and ZR-75-1, are well-suited for antihormone resistance studies as they are easily cultured and retain ER expression when treated with antihormones; they are routinely used in the laboratory and have produced more data of practical knowledge for patient care than any other breast cancer cell line (see final section). Figure 7 illustrates

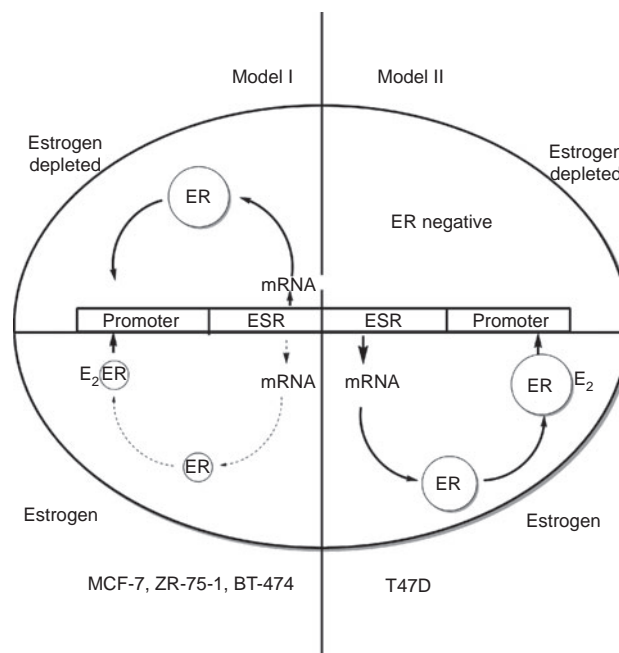


Figure 6 The diagrammatic representation of cellular ER regulation in media with or without estradiol (E_2). This diagram is based on the general responses to estrogen illustration by Western blotting in Figure 2 and presented in detail in [48]. Model I ER regulation (MCF-7, ZR-75-1, BT-474) has an upregulation of ER message and protein in an estrogen-depleted environment, but ER is down-regulated at the mRNA and protein level in the presence of estrogen. Model II ER regulation (T47D) has upregulation of ER message and protein in an estrogen-containing environment, but ER is not produced in an estrogen-depleted environment. Cells lose ER to become ER negative.

the lineages of different subtypes of MCF-7 cells maintained in the laboratory.

One such in vitro model illustrating the varied attributes of tamoxifen-resistant cells are the MCF-7 Lombardi Cancer Center (LCC) subclones (see Figure 7). The MCF-7:LCC1 variant represents an estrogen-independent breast cancer cell line obtained from in vivo selection in oophorectomized nude mice and re-cultured in vitro to become a stable cell line [50, 51]. Though estrogen-independent, the cells are still tamoxifen-sensitive [50]. When this cell line was selected for tamoxifen resistance in vitro, the MCF-7:LCC2 clone was created. MCF-7:LCC2 cells are stable, ER-positive, and respond to the pure antiestrogen, fulvestrant [52]. Along the same lineage, MCF-7:LCC9 cells were derived by selecting in vitro MCF-7:LCC1 cells for fulvestrant resistance, and subsequently, these cells exhibit cross-resistance to tamoxifen [53].

Another early antiestrogen-resistant variant of MCF-7 cells is the LY2 line. MCF-7:LY2 cells are resistant to LY117018, a potent antiestrogen related to raloxifene [54]. The LY2 cells also exhibit cross-resistance to tamoxifen and continue to be responsive to estrogen but with lower ER levels than MCF-7. The cell line was created by selection with increasing the concentration of LY117018 up to 1 μ M as MCF-7 cells became

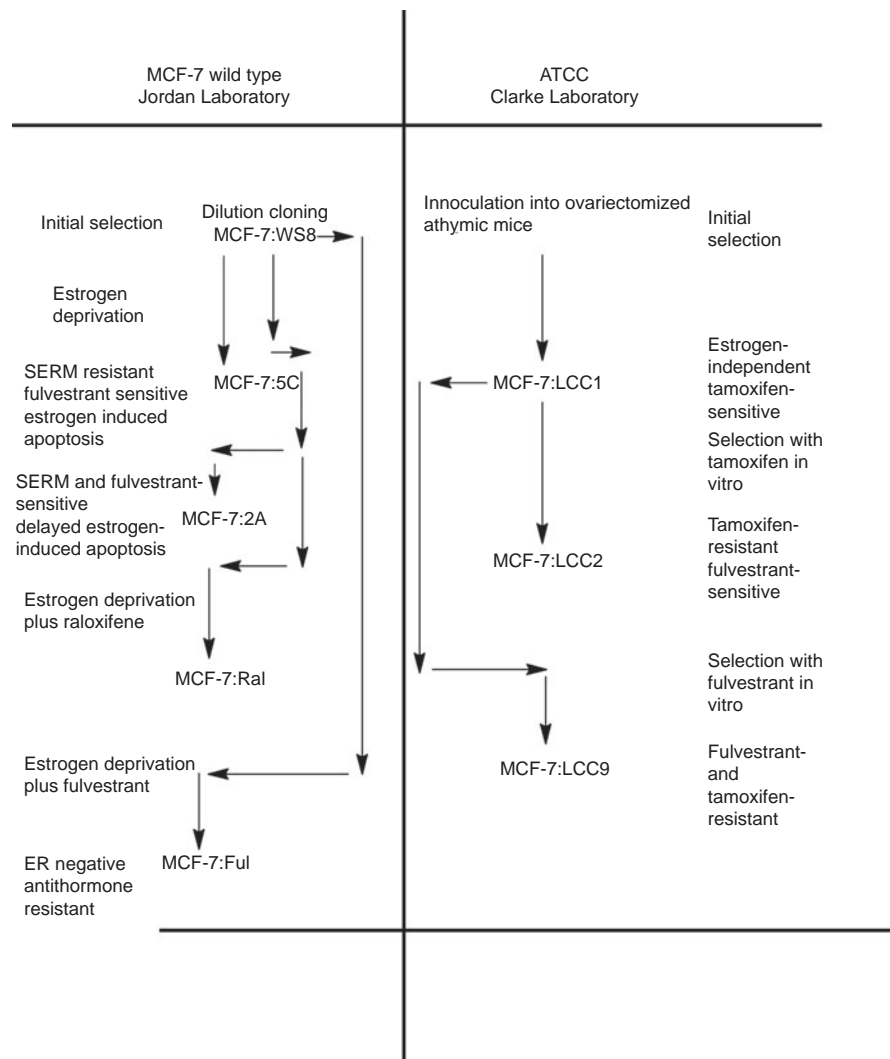


Figure 7 A flow diagram representation of the defined antihormone-resistant cell lines derived from MCF-7 cells. The Jordan Laboratory obtained original “Soule” MCF-7 cells from the Michigan Cancer Foundation as a gift from Dr. Dean Edwards who was then at the University of Texas. The Clarke Laboratory obtained MCF-7 cells from the ATCC cell collection. All cells are genotyped by DNA fingerprinting.

resistant [54]. A related MCF-7 raloxifene-resistant line MCF-7/RAL was created by growing MCF-7 cells in estrogen-free culture with 1 μ M raloxifene for over a year [55]. These cells grow in response to estradiol and raloxifene and are growth-inhibited by fulvestrant [56]. Most importantly, the cells exhibit an unusual apoptotic response to estradiol in vivo (see next section). The MCF-7/F cell line was established by culturing the parental MCF-7 cells in fulvestrant-containing estrogen-free media for 18 months. ER expression was lost, and the cells became resistant to all antihormone therapies [57].

Short-term estrogen deprivation causes distinct responses of MCF-7 cells in comparison to long-term (over 6 months) estrogen deprivation. These studies are important to mimic the early response of ER-positive breast cancer to aromatase inhibition. Culture of MCF-7 cells in media that is phenol red-free with charcoal-stripped serum (estrogen-free) causes immediate proliferation inhibition [42, 46]. Slowed proliferation

continues for about a month after estrogen removal, indicating the cells have not yet found adaptive or compensatory growth mechanisms. When stimulated with estradiol, the proliferation rate of these short-term estrogen-deprived cells increases, and antiestrogens again inhibit growth [42, 46]. Over time, MCF-7 cells deprived of estrogen eventually adapt their growth in estrogen-free media, losing their estrogen sensitivity, but antiestrogens continue to inhibit growth [43]. The ER is retained and expanded.

In 1995, Santen’s group hypothesized [58] that MCF-7 cells develop hypersensitivity to minute concentrations of estradiol (or indeed any available estrogen) after estradiol deprivation as a means of adapting to estrogen withdrawal and spontaneous growth. They noted that when MCF-7 cells are deprived of estrogen for 1–6 months, a 10-fold lower concentration of estradiol is needed for maximal growth, when compared to normally cultured MCF-7 cells. This model suggests an explanation for spontaneous growth that occurs after estrogen

withdrawal; that is, the breast cancer cells are hypersensitive to minute environmental concentrations of estrogen [58]. Indeed, this is a valid hypothesis as the estrogen-deprived cell population adapts by selecting any available cell to grow in the environment: a Darwinian model.

Long-term estrogen-deprived (LTED) MCF-7 cells form a stable cell line that has been used to investigate estrogen's effect on breast cancer cells over varied exposures and lengths of time. MCF-7:LTED cells, in contrast to their short-term estrogen-deprived counterparts, are able to grow despite lack of estrogen in the media and are growth inhibited by estradiol [43].

MCF-7:5C cells were developed by long-term estrogen withdrawal from the parental wild-type MCF-7 breast cancer cells [59, 60]. The ER in MCF-7:5C cells is wild type, and expression levels are similar to MCF-7 [59] (see Figure 2). This hormone-independent, ER-positive, PR-negative clonal population proved useful in representing the behavior of long-term estrogen-deprived breast cancer cells, that is, those of postmenopausal women decades after menopause or patients who have undergone long-term antihormone therapy, e.g., 5-year aromatase inhibitor treatment [60]. MCF-7:5C cells are unresponsive to 4-hydroxytamoxifen, and estradiol does not enhance growth [59, 60] but triggers estradiol-induced apoptosis [44].

The MCF-7:2A cell line is similar to the MCF-7:5C cell line and was generated from long-term estrogen withdrawal from MCF-7 cells. Uniquely, MCF-7:2A cells express two forms of the ER, a 66-kDa wild type and a 77-kDa mutant (see Figure 2) [48, 61]. The wild-type ER, expressed 4- to 10-fold higher than the mutant, is still functional, whereas the mutant ER, containing a repeat of exons 6 and 7 in the ER gene [62], can no longer bind estrogens nor antiestrogens. MCF-7:2A cells grow in estrogen-free media as they are estrogen-independent. In contrast to its parental cell line, the 2A cells show no response to estradiol during the first 7 days of treatment, then begin to die via apoptosis during week 2. Both tamoxifen and pure antiestrogens block growth in these cells [48, 61].

In search of other *in vitro* models illustrating antihormone-resistant breast cancer cells, the T47D cell line can offer additional information. T47D cells differ from MCF-7 cells in that their tumor suppressor protein p53 is mutated on one allele of the gene (194 Leu→Phe) [63]. Also, MCF-7 cells continually express ER, whereas T47D lose ER expression when estrogen is withdrawn for extended periods of time [64]. The T47D:A18 variant is ER positive and PR positive, derived from culturing the T47D cell line in estrogen-rich media [64]. They grow in response to estrogen and are inhibited by 4-hydroxytamoxifen [64]. T47D:C4 cells, in contrast, were established by culturing T47D cells in estrogen-free media [30, 64]. The parental cells are transformed into ER-negative, PR-negative cells, which are unresponsive to antihormone therapy [65].

To address mechanistic issues of antihormone resistance, T47D-r cells, also derived from the parental T47D line, were created to be resistant to fulvestrant [66]. Proteomic analysis was used to compare T47D vs. T47D-r cells to identify

38 proteins with significantly (2-fold up- or downregulation) different expression [66]. Furthermore, mRNA expression differed for 11 of the proteins. These data are evidence supporting the molecular and mechanistic changes that occur to T47D breast cancer cells as they become increasingly resistant to antiestrogens [66]. The T47Dco subclone is estrogen- and antiestrogen-resistant and expresses PR regardless of estrogen stimulation. Progestins inhibit proliferation of T47Dco cells [67]. Initially described as ER negative [67], it was subsequently shown that the cells express three mutant ERs that have no ability to bind ligand [68]. This cell line allows for extensive study on progestins' effect on breast cancer independently of estrogen, as well as on ER mutations as a mechanism of hormone resistance.

When ZR-75-1 cells are treated with tamoxifen for 6 months, both ER and PR levels decrease, but the antihormone is still able to impede the cancer growth. Tamoxifen resistance occurs after a year of tamoxifen treatment, as evidenced by the tamoxifen-resistant subclone ZR-75-9a1, a distinct ER-negative, PR-negative cell line [69]. Table 1 summarizes the discussed cell lines' subclones used for modeling ER-positive breast cancer cells *in vitro*.

Models of acquired antihormone resistance *in vivo*

Laboratory studies of endometrial cancer *in vivo* aided in the understanding of acquired resistance to tamoxifen. Estradiol significantly increases the growth rate of human ER-positive endometrial cancer transplanted into ovariectomized nude mice, while the growth rate of ER-negative endometrial cancer in this model is unaffected by estradiol treatment [70]. However, ER-positive endometrial tumors implanted in nude mice also grew more quickly in response to tamoxifen or estradiol treatment than the control-treated mice [71]. When medroxyprogesterone acetate (MPA) (a standard therapy for endometrial cancer) was administered to the tamoxifen-treated animals implanted with endometrial tumors, inhibition of growth was increased in comparison to the tamoxifen-treated tumors alone. In contrast, the growth of ER-negative endometrial cancer injected into athymic mice was unaffected by all treatments [71].

Subsequently, the human endometrial tumor EnCa101 was pivotal in enhancing knowledge of the target site specificity of tamoxifen, as well as by other similar triphenylethylene antiestrogens (e.g., clomiphene, trioxifene, nafoxidine) [72]. Athymic mice transplanted with both MCF-7 breast and EnCa101 endometrial tumors, and treated with either estradiol, tamoxifen, or a combination, demonstrated that estradiol increases the growth of both tumors. Tamoxifen, however, blocks breast cancer growth while enhancing the growth of endometrial cancer [16]. These data were rapidly translated to patient care [17], with breast cancer patients being given routine gynecological examinations to detect endometrial cancer that was slightly but significantly increased during adjuvant tamoxifen therapy. The target site-specific action of tamoxifen in breast and endometrium was hypothesized

Table 1 Various subclones generated from different ER-positive breast cancer cell lines.

Parental line	Subclone	How subclone was generated	Subclone's resistance	Reference
ZR-75-1	9a1	Long-term tam treatment	Tam	[69]
T47D	ER-negative	Estrogen withdrawal	Antihormones	[30]
T47D	-r	Long-term fulv treatment	Fulv	[66]
T47D	A18	Estrogen-rich culture	–	[64]
T47D	C4	Estrogen withdrawal	Antihormones	[65]
T47D	co	PR expression selection without estrogen	Estrogen, Antiestrogen	[68]
MCF-7	Ral	Long-term ral treatment	Ral	[55]
MCF-7	F	Long-term fulv treatment without estrogen	Fulv	[57]
MCF-7	5C	Estrogen withdrawal	Tam	[59, 60]
MCF-7	2A	Estrogen withdrawal	–	[61]
MCF-7	LY2	LY117018 selection	Tam, LY117018	[54]
MCF-7	LCC1	Estrogen withdrawal	–	[50, 51]
MCF-7	LCC2	Estrogen withdrawal, tam selection	Tam	[52]
MCF-7	LCC9	Estrogen withdrawal, fulv selection	Tam, Fulv	[53]

To simulate different scenarios of therapy and development of resistance to SERMs, cells were cultured in different environments to create stable cell lines. Fulv, fulvestrant; Tam, tamoxifen; Ral, raloxifene; Ref, reference number.

to be dependent on differential modulation of the estrogenic actions of tamoxifen in different target tissues [73]. The concept was supported by studies of antiestrogens with reduced estrogenic action. Keoxifene (subsequently called raloxifene) and LY117018 are less estrogenic in the rodent uterus and have less of an effect on EnCa101 growth stimulation [72, 74]. Further, ICI 164,384, as it is a pure antiestrogen with no intrinsic estrogenicity, did not stimulate EnCa101 tumor growth and was able to block tamoxifen-induced growth [15]. Clinical studies demonstrate that unlike tamoxifen, raloxifene [18] and fulvestrant [75] have no estrogen-like action in the human uterus.

MCF-7 models in vitro eventually evolved one step further toward clinical practice when they were adapted into models in vivo, which mirror more closely clinical care. Models in vivo create a new dimension to assess the importance of a functioning physiologic interaction between cancer cells, the interaction of angiogenesis, cellular metabolism, and respiration that are not created in cell culture. The first studies of MCF-7 cells implanted into nude mice were published in the 1980s. MCF-7 cells implanted into mice with intact ovaries, or simultaneously with estrogen into ovariectomized mice, grew in an estrogen-dependent manner [37].

In the 1980s, transplanted models of MCF-7 human breast cancer into athymic mice were used to investigate the unique aspects of acquired resistance to SERMs. Tamoxifen acts as a competitive inhibitor of estradiol-stimulated growth, i.e., the action of tamoxifen as an antitumor agent is reversed by increasing the dose of estradiol [76]. Similarly, months of tamoxifen therapy do not destroy implanted MCF-7 tumors [77, 78], as estrogen can reactivate tumor growth. Eventually, acquired resistance to tamoxifen occurred after 4 months of treatment, wherein neither tamoxifen nor estrogen deprivation could produce significant tumor regression [79]. Breast tumors then grew despite tamoxifen treatment demonstrating that acquired resistance to antihormone therapy had developed.

However, a similar study came to a different conclusion; MCF-7 tumors grew in the athymic mouse not despite tamoxifen therapy but because of tamoxifen therapy [80]. When the MCF-7 tumors resistant to tamoxifen were transplanted into new athymic animals, these ER-positive, PR-positive tumors were found to grow in response to either estradiol or tamoxifen treatment. It is also noteworthy that the tamoxifen-stimulated tumors expressed twice the level of ER when compared to their estradiol-stimulated counterparts [80]. A survey of other steroidal and non-steroidal antiestrogens demonstrated that tamoxifen-stimulated growth is dependent on the estrogen-like actions of tamoxifen. Less estrogenic agents do not increase the growth of acquired tamoxifen resistance in MCF-7 tumors [81]. There is cross-resistance with other antiestrogens, e.g., toremifene or raloxifene [82, 83] but not fulvestrant. Overall, this model mimics the development of acquired resistance to tamoxifen during the treatment of metastatic breast cancer. The tumors become resistant to therapy in about 2 years.

Many of the previously discussed MCF-7 subclones have been examined in animal models. When the MCF-7/RAL cells are transplanted into athymic ovariectomized mice, they are able to form tumors when treated with either estradiol or raloxifene. Eventually, after about 8 months of re-transplantation, the tumors grow only in response to raloxifene and are inhibited by estradiol [56].

MCF-7/LCC1 cells are estrogen-responsive and tamoxifen-sensitive in vivo. MCF-7/LCC2 cells, on the other hand, behave estrogen-independently in vivo. They continue to exhibit tamoxifen resistance in vivo as they do in vitro [52]. The MCF-7/LCC9 cell line, consistent with its in vitro action, can form tumors in the athymic ovariectomized mouse and are unresponsive to fulvestrant [53].

Similarly, MCF-7 cells with acquired resistance to tamoxifen (MCF-7:Tam) in vivo implanted in athymic ovariectomized mice grow in response to tamoxifen or estradiol, but the steroidal antiestrogen RU 39,411 or ICI 164,384 inhibit growth [81]. However, long-term transplantation of MCF-7:Tam tumors into athymic mice eventually results in a

change in response to physiologic estradiol with rapid tumor regression [84, 85]. Similarly, MCF-7:5C cells injected into athymic ovariectomized mice undergo apoptosis when treated with estradiol, causing complete tumor regression [44]. This unusual change in the biology of the tumors will be revisited in the next section.

T47D cells have also been examined *in vivo* to evaluate the role of SERMs to create acquired antihormone resistance. T47D cells transplanted into athymic ovariectomized mice can generate tumors in response to estradiol, and tamoxifen can inhibit this estrogen-stimulated growth. However, after high-dose (1.5 mg daily) tamoxifen treatment, the tumor cells become tamoxifen-resistant after about 8 weeks, wherein tamoxifen begins to stimulate tumor growth [86]. The T47D cells giving rise to tamoxifen-stimulated tumors produce a subtype of T47D cell named T47D:Tam. Other SERMs, arzoxifene and LY117018, did not increase growth of T47D:Tam tumors *in vivo*; likewise, arzoxifene and LY117018 did not increase the growth of estradiol-stimulated T47D tumors either. This indicates a lack of cross-resistance between tamoxifen and the other antiestrogens in T47D cells *in vivo* [87].

In addition to SERM studies, models *in vivo* also examined the effect of aromatase inhibition on ER-positive cell lines. In 1994, nude mice were injected with MCF-7 cells transfected with the human aromatase gene to study the action of aromatase inhibitors *in vivo* for the treatment of breast cancer [88]. In the normal nude mouse, tumors grew in response to ovarian estrogen and were inhibited by aromatase inhibitors and tamoxifen. The aromatase substrate, androstenedione, was administered to the ovariectomized mice in order to model human disease as mice express no androgen precursor. Ovariectomized nude mice injected with aromatase-transfected MCF-7 cells grew tumors utilizing estrogen produced through the aromatization of androstenedione via the aromatase pathway. Aromatase inhibitors (4-hydroxyandrostenedione and CGS 16949A) and tamoxifen were able to block the tumor growth. This latter model represents postmenopausal women whose tumors grow not in response to ovarian estrogen, but estrogen generated through the aromatization of androgens found primarily in the adipose tissue. MCF-7 cells transfected with the aromatase gene and injected into ovariectomized mice were inhibited better with the combination treatment of fulvestrant and anastrozole than either agent alone. This suggests the targeting of both aromatase and the ER for better treatment of postmenopausal breast cancer patients [89]. These studies provide a rationale behind aromatase inhibitors' efficacy in the clinical setting [88].

Laboratory models set the stage for intense evaluation of antihormone-resistant breast cancer cells. By continuing investigation of mechanisms of resistance, many unique and sometimes paradoxical effects of hormones and antihormones on ER-positive breast tumors have been discovered. The finding that an estrogen and an antiestrogen could eventually stimulate breast cancer growth demonstrated the unique qualities of acquired resistance to SERMs [80]. The aforementioned individual findings now began to form models for

the evolution of acquired resistance that can not only be interrogated in the laboratory but also applied to clinical care.

Evolution of acquired antihormone resistance

Based on laboratory evidence from both individual reports and studies of up to a decade, the evolution of acquired resistance to SERMs can now be described in distinct phases following long-term SERM treatment and long-term experiments *in vitro* and *in vivo* (Figure 8) [90, 91]. The evolution (Figure 8) of acquired resistance occurs after an initial period of therapeutic success where antiestrogenic activity predominates, and the SERMs are competitive inhibitors of estrogen-stimulated tumor growth in athymic mice [76, 77]. The therapeutic phase of SERM action can be maintained for a year or two (at most), but eventually, tumors start to grow despite continued tamoxifen [79]. However, these tumors can be re-transplanted into other tamoxifen-treated ovariectomized athymic mice [80]. Paradoxically, both physiologic estradiol and tamoxifen (there is cross-resistance with raloxifene and toremifene) [82] can then cause growth, indicating Phase I resistance. The pure antiestrogens ICI 164,382 and fulvestrant block Phase I growth with either tamoxifen or estradiol. A similar form of acquired resistance to tamoxifen occurs with the T47D breast cancer cell line [86, 87]. This type of acquired resistance is characteristic of resistance to tamoxifen during the treatment of metastatic ER-positive breast cancer and is why either fulvestrant or an aromatase inhibitor is effective second-line therapeutic agent in the clinic [92, 93]. The laboratory principles are illustrated in Figure 8.

However, these laboratory data are inconsistent with the successful adjuvant treatment of node-positive and node-negative ER-positive breast cancer with 5 years of tamoxifen [7]. In fact, not only is tamoxifen effective during adjuvant

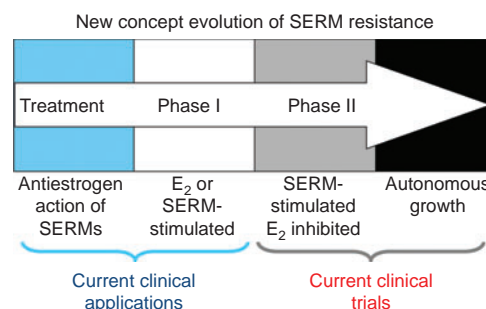


Figure 8 Evolution of acquired SERM resistance. After long-term treatment with SERMs (1–2 years *in vivo*), initially responsive ER-positive tumors become resistant to treatment and are stimulated by SERMs (Phase I of resistance) as well as by E₂. After long-term transplantation into SERM-treated animal (5+ years), breast tumor growth is inhibited by E₂, though still stimulated by SERMs (Phase II of resistance). A stylized representation of MCF-7 tumor growth is illustrated in Figure 9. This process with SERMs *in vivo* is replicated with estrogen deprivation with MCF-7 breast cancer cells *in vitro*; cells initially start to grow spontaneously, but estrogen still induces growth (hypersensitivity). This is Phase I. Long-term estrogen deprivation causes spontaneous growth in culture but apoptosis with physiologic estrogens both *in vitro* and *in vivo* (Phase II).

therapy but also effective at maintaining recurrence-free survival and reducing mortality by 30% from the 10 years following tamoxifen being stopped. Laboratory studies have now provided an insight into this clinical advance.

Repeated transplantation of tamoxifen-resistant tumors into subsequent generations of tamoxifen-treated athymic mice results in a change in the clonal selection of tumor cells. Not only do the tumors remain tamoxifen-dependent for growth over a 5-year period but also the constant exposure to tamoxifen treatment changes the tumor response to estradiol from being a survival signal to an apoptotic trigger. Tumor regression occurs in response to physiologic estrogen, and this has been proposed as a mechanism to explain the decreasing mortality of tamoxifen-treated patients following adjuvant tamoxifen [84, 85]. In other words, short-term adjuvant tamoxifen only pushes acquired resistance into Phase I resistance where estradiol is still a growth stimulator once tamoxifen is stopped. In contrast, longer tamoxifen treatment forces clonal selection into Phase II resistance where apoptosis occurs upon exposure to a woman's own estrogen. This is illustrated when a comparison between Figure 9A and 9B is made. Indeed, it was proposed that as tumors that regress and subsequently regrow in response to physiological estrogen can again respond to subsequent antihormone treatments, then this could be applied in the clinic [85]. This experiment has recently been reported in a clinical study by Ellis et al. [95].

The evolution of cell populations to long-term antihormone therapies has been replicated with raloxifene in a 10-year study in vivo [56]. The reason for doing this is that raloxifene will be used indefinitely to prevent osteoporosis [19] and breast cancer [21]. The same evolution of acquired resistance occurs with the development of Phase I and Phase II raloxifene resistance, characterized by Phase I resistance with estradiol- or raloxifene-stimulated tumor growth, and Phase II resistance characterized by estradiol-induced tumor regression. It is perhaps relevant to point out that MCF-7 cells exposed to both raloxifene and estrogen deprivation in vitro rapidly advance to Phase II resistance with estradiol-induced apoptosis in vivo [55].

Additionally, there are a couple of other clinically relevant points that can be made about acquired SERM resistance in the laboratory. The T47D cell line advances to Phase I tamoxifen resistance but does not progress to Phase II. The fact that T47D cells have mutant p53 may be relevant as estrogen-induced apoptosis does not develop.

The pure antiestrogen fulvestrant is an excellent antiestrogen/antitumor agent in the laboratory, but results have been disappointing clinically until the recent successful use of twice the recommended dose [94]. Laboratory studies with Phase II tamoxifen-resistant tumors grown in athymic mice suggest that the second-line use of fulvestrant in an environment of physiologic estrogen is destined to fail and, in fact, cause enhanced tumor growth [96]. The reason for this is unknown.

The fact that aromatase inhibitors are now the adjuvant treatment of choice for postmenopausal patients with ER-positive breast cancer makes an examination of acquired resistance mandatory. Suffice to say that the principles first described for SERMs are true for aromatase inhibitors and the development of acquired resistance to estrogen deprivation in vivo [97–99] and in vitro [32, 42–44, 100].

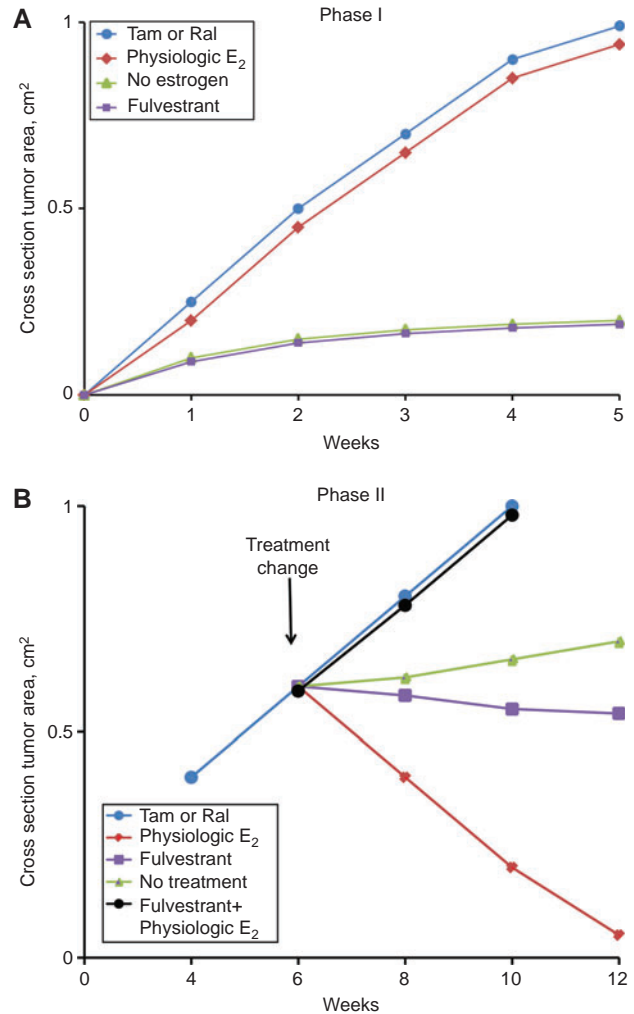


Figure 9 Diagram of the growth rates of MCF-7 tumors during the evolution of drug resistance to SERMs. (A) During Phase I SERM resistance, tumors transplanted into athymic mice grow in response to either a SERM, tamoxifen (Tam) or raloxifene (Ral), or estrogen, but no estrogen (equivalent to the use of an aromatase inhibitor used clinically after Tam resistance occurs) or fulvestrant does not support growth (fulvestrant is used in this indication as a second-line therapy). (B) During Phase II SERM resistance, tumors transplanted into athymic mice treated with SERMs now grow with a SERM (Tam or Ral). No treatment (equivalent to an aromatase inhibitor clinically) causes growth to slow, as does administering fulvestrant, but physiologic estradiol (E₂) causes dramatic apoptosis and tumor regression. Paradoxically, physiologic E₂ plus fulvestrant actually causes tumor growth. The low concentration of fulvestrant cancels out the apoptotic effect of E₂ thereby redirecting E₂ as a growth signal, but higher concentrations of fulvestrant now have effective antitumor effects. This is now noted clinically [94].

Mechanisms of acquired antihormone resistance

Breast cancer can be resistant to antihormones in varied ways. As previously noted, intrinsic resistance can occur de novo wherein antihormone therapy generates no disease regression. This occurs in ER-negative tumors, as well as in some subgroups of ER-positive tumors. However, we will focus on

the mechanisms involved in the evolution of acquired antihormone resistance. Acquired resistance to antihormone therapy can be caused by three main mechanisms to be discussed here: loss of ER function, aberrant growth factor signaling, and estrogen-induced apoptosis.

Loss of ER function as a mechanism of acquired antihormone resistance

Experiments *in vitro* provide an initial platform for studying the mechanisms of acquired antihormone resistance. First, if the ER in breast cancer cells is altered, the effects of antihormones will be altered accordingly. If ER expression is lost, the whole mechanism of endocrine therapy will be undermined; ER-mediated actions will no longer contribute to proliferation or apoptosis. Similarly, if ER is mutated in such a way that no longer binds its ligands, resistance will occur. Nonetheless, ER mutation is not a major factor in drug resistance but one example that has provided insight into ER modulation of anti-estrogen action [101–104].

If the promoter regions of ER target genes are hypermethylated during acquired resistance, transcription of ER target genes is again blocked, abrogating antihormone efficacy *in vitro* [105]. Coupling of ubiquitin conjugation to ER degradation (CUE) domains are approximately 50 amino acids long and bind monoubiquitin molecules used in trafficking and ubiquitylation [106]. CUE domain-containing protein-2 (CUEDC2) is shown to have an inverse correlation with ER protein expression in breast cancer cells *in vitro*. High levels of CUEDC2 protein expression correlate with tamoxifen resistance, probably due to loss of ER via the ubiquitin/proteasome pathway [107].

If the ER is inactivated because of histone methylation or deacetylation, treating breast cancer cells that have acquired resistance to antihormones with a histone deacetylase (HDAC) inhibitor can re-activate the ER. This concept has been illustrated using ER-negative MDA-MB-231 wherein an HDAC inhibitor generates both ER and aromatase expression. Letrozole can then be used as effective treatment [108], suggesting a potential treatment mechanism for ER-positive cells that have lost ER expression during acquired resistance. Loss or reduction of ER as a primary cell survival pathway can also be replaced by an increase in the mosaic of growth factor signaling pathways. These pathways can modulate and subvert steroid hormone receptor synthesis and action [109, 110].

Growth factor signaling as a compensatory mechanism of survival

Growth factor signaling and ER crosstalk are consistent mechanisms by which acquired resistance to antihormones develops. It provides the breast cancer cells a means of escape from suppressive signaling and a way to continue proliferation. Growth factors may be able to contribute enough proliferative signal to drive ER target gene transcription even without normal ER ligand [111]. Growth factor signaling contributes indirectly to ER function, both genomically and non-genomically [111].

An important mechanism for bypassing antihormone-induced apoptosis is through increased expression of membrane receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), fibroblast growth factor receptor (FGFR), and HER2. These membrane receptors can activate not only the ER signaling pathway [112] but also the MAPK and AKT signal transduction pathways through increased phosphorylation of p42/44. This is demonstrated *in vitro* using MCF-7:LTED cell growth inhibition by IGFR knockdown [113]. OSI-906, an IGFR tyrosine kinase inhibitor, prevents MCF-7:LTED growth both *in vitro* and *in vivo* [113].

When EGFR is transfected into ZR-75-1 cells, the cells become estrogen-independent. These cells become ER negative when tamoxifen is introduced and continued to grow using EGF and its receptor, indicating a possible growth mechanism for antihormone-resistant breast cancer cells [114]. Further, ZR-75-1 cells treated with a 5-azacytidine (a DNA methylation inhibitor used to study influence of epigenetic changes on acquired estrogen independence) develop estrogen independence when grown in estrogen-free media, increasing their HER2 and EGFR expression. Growth of these antihormone-resistant cells can be slowed by an anti-EGFR antibody, indicating a crucial role of EGFR and growth factor signaling in the progression of antihormone resistance in ZR-75-1 cells [115]. When EGF-stimulated growth was measured in MCF-7 cells, it was not able to be blocked by tamoxifen, 4-hydroxytamoxifen, nor ICI 164,384, suggesting an important growth factor influence on their proliferation [116]. Further, breast cancer cells with amplified FGFR show increased resistance to 4-hydroxytamoxifen *in vitro*, reversible with FGFR-targeted siRNA, indicating a mechanism driving endocrine resistance [117].

If cancer cells are using downstream signaling pathways to continue their growth independent of ER, then blocking key signaling molecules could reveal additional mechanisms of escape. Antagonists of downstream ER signaling pathway proteins, such as mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K), provide potential targets to prevent breast cancer growth after antihormone resistance occurs. The combination of tamoxifen and the mTOR inhibitor RAD001 have an additive effect on MCF-7 cells, together blocking tumor growth *in vitro* better than either agent alone [118], identifying mTOR as an important target to delay the development of antihormone resistance.

Breast cancer cells that have acquired letrozole resistance highly overexpress the growth factor progranulin when compared to their letrozole-sensitive counterparts *in vitro* [119]. Progranulin is shown in the laboratory to cause breast cancer cells to acquire letrozole resistance, and knocking down this growth factor can confer letrozole sensitivity to cells that had acquired letrozole resistance, thereby blocking their proliferation [119]. This example again demonstrates the complexity and flexibility of breast cancer cells to utilize growth factor signaling for survival after long-term antihormone therapy [119].

Long-term estrogen-deprived ER-positive breast cancer cells transfected with the human aromatase gene were studied in ovariectomized athymic nude mice to elucidate

mechanisms of acquired resistance to aromatase inhibitors in vivo. Similar concepts emerge in vivo as have been described in vitro. Letrozole-resistant tumors express decreased levels of ER compared with letrozole-sensitive tumors in vivo and an increase in HER2 (six-fold) and EGFR tyrosine kinase receptors and their downstream signaling proteins (e.g., MAPK), suggesting a shift in signaling pathways away from ER [99, 120–123]. Inhibiting these tumors with the anti-HER2 monoclonal antibody trastuzumab restores letrozole sensitivity [123, 124] by downregulating HER2 and restoring ER expression [108]. This indicates that letrozole-resistant ER-positive tumors utilize HER2 signaling to survive despite therapy. HER2 and ER expressions were shown in vivo to correlate inversely with one another; that is, when HER2 is inactivated by trastuzumab ER expression increases, and the cells become re-sensitized to antihormones and aromatase inhibition [99, 121]. EGFR inhibitors are also able to restore letrozole sensitivity [122].

Proteins involved in the MAPK signaling pathway, p-Raf, p-Mek1/2, and p-MAPK, are increased in tumors in vivo that have acquired resistance to letrozole [122, 123, 125], suggesting the activation of aberrant signaling for compensatory proliferation after long-term aromatase inhibition. Blocking ER with fulvestrant simultaneously with the PI3K inhibitor wortmannin is more effective than antihormone alone, suggesting that the pathway involving PI3K provides a means of growth escape to long-term antihormone-treated breast cancers [126].

Growth factors, e.g., the nuclear coactivator amplified in breast cancer-1 (AIB1, also called SRC-3 and NCoA-3) can activate the ER pathway during antihormone treatment. In the clinical setting, high levels of AIB1 expression in tamoxifen-treated tumors is associated with worse disease-free survival for breast cancer patients, illustrating the importance of AIB1 in the resistance pathway [127]. AIB1 exerts control over many of the growth factor signaling pathways relevant to acquired antihormone resistance, such as EGFR, HER2, PI3K, and mTOR, and interacts with many proteins associated with transcription, cell cycle regulation, and protein degradation [128, 129].

Estrogen-induced apoptosis mechanisms during acquired Phase II resistance

The most significant aspect of the evolution of antihormone resistance is the drift toward reconfiguring signaling networks to make the cell survive with no estrogen, but this creates a vulnerability to estrogen-induced apoptosis. After 5 years of treatment with antihormones, the sophisticated growth pathways become sensitive and paradoxically collapsed by estrogen, once a growth and survival signal. Clinically in the past, women with breast cancer have been successfully treated with high-dose estrogen therapy [130, 131]. This was the first effective chemical therapy for any cancer and was the standard-of-care before tamoxifen [132]. Investigation has sought to uncover mechanisms by which apoptosis occurs in Phase II-acquired resistance and how estrogen makes this switch in signaling.

B-cell lymphoma 2 (Bcl-2) is a signaling molecule expressed in 40%–80% of primary breast cancers that functions to prevent apoptosis [133], thereby contributing to malignancy and resistance. It acts as an antiapoptotic signal in long-term estrogen-deprived ER-positive breast cancer cells [134] to subvert estrogen-induced apoptosis. Inhibition of Bcl-2 via siRNA in vitro confers caspase-7 and caspase-9 activation and causes the cells to be synergistically sensitive to estrogen-induced apoptosis [134], making Bcl-2 an interesting therapeutic target. Bcl-2-interacting killer (BIK) regulates calcium release from the endoplasmic reticulum that triggers downstream mitochondria-mediated apoptosis, also inhibiting Bcl-2. High levels of BIK's inhibitory chaperone, GRP78, in ER-positive breast cancer cells, prevents apoptosis and causes endocrine resistance [135], thereby asserting itself as another potential therapeutic target.

Studies of varied ER-positive breast cancer cells began to investigate the unique properties of physiologic estrogen that causes tumor regression in postmenopausal women [43]. Santen's group showed in 2001 [43] estrogen-independent growth of MCF-7:LTED cells and significant reduction of tumor growth when treated with estradiol. Using annexin V staining and Western blot analysis, the experiments demonstrated induction of FasL, a death receptor ligand associated with the apoptosis cascade, when cells were treated with estradiol [43]. This finding established the notion of estrogen-inducing Fas-mediated apoptosis in LTED breast cancer cells. Apoptosis via the Fas/FasL pathway was increased 7-fold in the estradiol-treated LTED breast cancer cells when compared to the vehicle-treated LTED cells [43]. Fas mRNA and protein were also increased in MCF-7:Tam tumors in vivo, correlated with decreases in NF- κ B expression. The laboratory experiment showed that increased Fas signaling and simultaneous suppression of NF- κ B's antiapoptotic signaling may be characteristic of estradiol-induced apoptosis [96].

Estrogen-induced apoptosis can also originate through the intrinsic mitochondrial apoptosis pathway, when cytochrome C is released from the mitochondria [44]. This is shown in the laboratory using MCF-7:5C cells in vivo [44]. MCF-7:5C cells injected into ovariectomized athymic mice exhibited increased apoptotic protein (e.g., Bax, Bim, p53) expression and tumor regression when treated with estradiol [44].

In tamoxifen-stimulated (Phase II resistant) MCF-7 xenografts, fulvestrant can reverse estrogen-induced apoptosis, stimulating growth and expression of phosphorylated HER2, HER3, p-ERK1/2, and p-GSK3 α and β proteins [136]. Pertuzumab blocks the interaction of p-HER2 and HER3 and is able to decrease tumor growth in this model in vivo, suggesting that fulvestrant stimulation of antihormone-resistant ER-positive breast cancers depend not on ER or ER target genes, but on the HER2/HER3 signaling pathway [136].

Additionally, AIB1 is required for estrogen-induced apoptosis in MCF-7:5C cells in vitro. The Wellstein group found that AIB1 is involved in signaling pathways that encourage apoptosis in this context, most prominently through associations with G-protein-coupled receptors, PI3K, Wnt, and Notch signaling pathways [129]. MCF-7 gene expression was examined for the WS8 (wild type), 5C, and 2A-derived

cell lines to examine differences in gene regulation during Phase II estrogen-induced apoptosis [100]. For the cell line most sensitive to estrogen-induced apoptosis (MCF-7:5C), genes associated with estrogen signaling, endoplasmic reticulum stress, and inflammation were upregulated, along with apoptotic genes such as BIM and caspase-4, in comparison to WS8 and 2A cells. Analysis of the gene regulation and protein expression indicates that estrogen-induced apoptosis is induced through an inflammatory response in the breast cancer cells, inducing proinflammatory genes (e.g., IL, IFM, arachidonic acid) [100]. The aforementioned examples allow translational research to apply laboratory-revealed mechanisms of acquired resistance to antihormones toward treatment strategies for overcoming or preventing such resistance in ER-positive breast cancer.

Clinical translation via cell models of ER-positive breast cancer

Laboratory models *in vitro* and *in vivo* are the invaluable link to clinical translation and enhanced patient survivorship. During the past three decades, the ER-positive breast cancer cell line MCF-7 has been indispensable in this process not only to test therapeutic strategies but also to advance our understanding of hormone-dependent cancer growth [32]. The MCF-7 cell line was the first hormone-responsive breast cancer cell line used effectively to decipher hormone action in breast cancer [32]. Additionally, the ER from MCF-7 cells was prepared on an “industrial scale” to prepare the first monoclonal antibodies [137, 138]. These antibodies are now used ubiquitously to determine the ER status of a patient’s tumor by immunohistochemistry [139–142] or flow cytometry [143–145]. However, it was the acquisition of monoclonal antibodies that permitted the cloning and sequencing of the human ER [146–148]. This advance has had a major impact on our understanding of the structure-function relationships of ER-mediated cell regulation.

The availability of ER-positive breast cancer cells and the development of models to test therapeutic strategies continue to play an essential part in the development of clinical trials. By way of example, we will close by considering the role of the MCF-7 cell line in patient care. To set the scene, we will place the comments in the context of current clinical practice. There are two therapeutic scenarios to consider: disease in the premenopausal patient and disease in the postmenopausal patient.

Premenopausal women who present with ER-positive breast tumors are generally prescribed combination cytotoxic chemotherapy with 5 years of adjuvant tamoxifen treatment, while postmenopausal women with ER-positive breast cancer are likely to receive an aromatase inhibitor. If these antiestrogenic approaches fail to prevent recurrence, fulvestrant is used as a second-line antihormone treatment [149].

The strategy of targeting the ER in the tumor micrometastases with long-term adjuvant tamoxifen was created using the 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat

mammary carcinoma model [1, 3, 150]. The first specific aromatase inhibitor, 4-hydroxyandrostenedione (formestane) was compared and contrasted to tamoxifen in the DMBA-induced rat mammary carcinoma model [151–153], but with the development of the model of estrogen-simulated MCF-7 tumors grown in athymic mice in the early 1980s [37, 38], the DMBA model was discarded. Initial studies in the athymic mouse model [77] only served to confirm the previous results in the DMBA model, but the breakthrough with the MCF-7 model really occurred with the discovery of the evolution of drug resistance to either tamoxifen (or indeed any SERMs) or aromatase inhibitors. We will consider several examples of progress using models of resistance in available breast cancer cell lines that are changing patient care.

The discovery that *in vivo*-acquired tamoxifen resistance is unique, as the tumors grow with either tamoxifen or physiologic estrogen [80], recreated a new dimension to consider in therapeutics: the tumor was amplifying the weak estrogen-like properties of tamoxifen by cell selection. An antiestrogenic strategy of no estrogen (an aromatase inhibitor) or an antiestrogen with no estrogen-like properties was required. The genesis and development of fulvestrant, the injectable long-acting pure steroidal antiestrogen is long, dating back to the mid-1970s, but only now is the clinical community able to apply the drug optimally for appropriate patient care [94].

The idea for studying the therapeutic value of 6,7-substituted estradiol analogs was started through a joint research scheme between ICI pharmaceutical division and Leeds University. The idea was to develop a cytotoxic carrier molecule based on the binding of estradiol to ER that would invariably target and destroy ER-positive metastases [154]. The last compound tested in the series was a 7-substituted ($-\text{CH}_2-$)₁₀ chain with the alkylating function on the end. This was based on the knowledge from Roussel Uclaf chemists who had made resin columns to extract and purify the ER [155]. The 7-substitution was an appropriate substitution to retain ER binding. The project to discover ER-targeted cytotoxic agents was abandoned, but subsequently, and independently, scientists at ICI pharmaceuticals discovered the merits of this class of molecules to create a “pure” antiestrogen [156]. The lead compound, ICI 164,384, first tested successfully in the tamoxifen-stimulated MCF-7 tumor athymic mouse model [81], provided the reassurance necessary for the clinical development of fulvestrant [47] or an aromatase inhibitor as a second-line agent following the failure of tamoxifen [92, 93]. The clinical results mimicked the animal data.

Osborne’s group made the important discovery that transfection of the HER2/neu gene would enhance and accelerate the development of resistance in MCF-7 cells to tamoxifen [49]. This has had important implications for the selection of breast cancer patients for tamoxifen treatment. Indeed, it is the important interplay and interaction of the ER and growth factor receptor pathways that is currently a major focus of translational research. The question has become, “what are the mechanisms and changes that occur in breast cancer cell populations that cause acquired resistance?” Once this

question is answered, it will be followed by a different question of, “how do we use the knowledge to delay the process and improve survivorship?” A clinical trial was launched in 2009 comparing lapatinib, a HER2 tyrosine kinase inhibitor, with letrozole vs. letrozole alone in postmenopausal hormone receptor-positive patients who have acquired tamoxifen resistance [157]. Lapatinib increases progression-free survival in these patients better than the aromatase inhibitor alone, illustrating a compensatory mechanism of antihormone-resistant cells via HER2 after tamoxifen failure [157]. There are ongoing preclinical and clinical trials investigating the EGFR pathway as a growth mechanism after acquired resistance, comparing antihormone treatments, such as tamoxifen and aromatase inhibitors, with and without EGFR inhibitors, such as gefitinib and erlotinib [158, 159].

Breast cancer cells that have acquired resistance to anti-estrogen therapy are shown to remain sensitive to therapies targeted against the PI3K pathway [160]. Signaling molecules in the PI3K pathway are frequently mutated in antihormone-resistant ER-positive breast cancer and comprise a targetable pathway to inhibit for effective therapy [160]. Multiple Phase I and Phase II prospective randomized trials focused on combinations of PI3K pathway inhibitors (e.g., everolimus, trastuzumab, lapatinib, gefitinib, enzastaurin, tipifarnib, BMS-754807, IMCA12, AMG479) and antihormone treatments (e.g., letrozole, exemestane, tamoxifen, anastrozole, fulvestrant) are underway [160] and predicted to provide valuable information.

The encouraging study of mTOR inhibitors in antihormone resistance has advanced to a successful Phase II trial comparing the effectiveness of letrozole, an aromatase inhibitor, treatment alone vs. letrozole plus the mTOR inhibitor, everolimus, in patients with ER-positive breast cancer. The results [161] demonstrate increased response rates for the combination arm, which has prompted the initiation of a Phase III clinical trial comparing everolimus in combination with exemestane, a different aromatase inhibitor, for postmenopausal women with ER-positive breast cancer resistant to other aromatase inhibitors [162, 163].

Brodie's group has advanced knowledge of the development of acquired resistance to aromatase inhibitors. Fulvestrant (to destroy the ER) plus an aromatase inhibitor is superior to either strategy alone [89], and trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen [164]. Each of these strategies has been addressed in clinical trials [165–167] recruiting patients with ER-positive tumors in late-stage breast cancer, but it will be in the adjuvant setting that most gains may occur for patient survivorship. Osborne's group [158, 168] has independently pioneered the strategy of using multiple inhibitors of the growth factor receptor family in combination with either estrogen deprivation or tamoxifen therapy, and these strategies are moving into clinical trial.

However, it is the laboratory knowledge derived from the evolution of acquired resistance to long-term antihormone therapy that is providing an insight into past clinical research and future opportunities. All MCF-7 or T47D laboratory models for SERM resistance in vivo develop acquired

resistance within a year or two. This is consistent with the endocrine treatment of metastatic breast cancer but does not explain the remarkable success of 5 years adjuvant tamoxifen to create a 30% decrease in mortality, not only during therapy but sustained for 10 years after therapy stops [7]. The treatment of micrometastatic disease with tamoxifen is clearly different than treatment of established tumors. A breakthrough occurred in the early 1990s with the finding that three repeated transplantations of small MCF-7 tumor pieces into subsequent generations of tamoxifen-treated athymic mice for more than 5 years exposes a vulnerability to the tumor cells that rapidly die during physiologic estrogen treatment [84, 85]. This phenomenon was originally advanced [84] to explain the sustained antitumor action of tamoxifen when adjuvant treatment is stopped. It was suggested that women's own estrogen causes apoptosis in micrometastases during Phase II of acquired resistance. Subsequent studies in vitro with estrogen-deprived MCF-7 breast cancer cells demonstrated estradiol-induced apoptosis [43, 44].

Based on these studies with MCF-7 cells alone, clinical trials have demonstrated the effectiveness of both high- and low-dose estrogen therapy to treat breast cancer following the development of acquired resistance to antihormone therapy in metastatic disease [95, 169]. The approach [84, 170] is now being applied indirectly to adjuvant clinical trials of long-term adjuvant therapy (Study of Letrozole Extension), where it is anticipated that a 3-month drug holiday per year for 5 years may reduce recurrence rates during letrozole adjuvant therapy. This is the same principle that is now applied to explain [171] the efficacy of low-dose estrogen replacement alone to reduce the incidence of breast cancer in women with a median of 20 years past their menopause (i.e., long-term estrogen deprivation) [172].

For the future of research in cellular models of breast cancer and acquired resistance to antihormone therapy, there are four new developments. First, new primary breast cancer cell lines are being developed and tested both in vivo and in vitro for drug sensitivity. Second, a huge pool of human breast cancer cell lines has been interrogated for drug sensitivity and pathway analysis completed to procure new clinical strategies for treatment [173, 174]. Third, signatures have been created to define acquired drug resistance to tamoxifen in existing breast cancer cell lines [117, 175] that can be applied to clinical trial. Finally, new methodologies are now available to enrich for breast cancer stem cells and expanding this populations for drug sensitivity testing [176]. Should the future of the “many” new cell systems from primary tumors deliver the promise achieved by the “few” cell lines in the past, then there is every reason to believe that enormous progress will occur in the successful treatment and prevention of breast cancer in the coming decades.

Acknowledgments

This work (VCJ) was supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence, subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409, the Susan G Komen For The Cure

Foundation under Award number SAC100009, and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

References

- Jordan VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008;44:30–8.
- Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clin Cancer Res* 2003;9:1980–9.
- Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. *Eur J Cancer* 1975;11:205–6.
- Brodie A. Aromatase and its inhibitors – an overview. *J Steroid Biochem Mol Biol* 1991;40:255–61.
- Chumsri S, Howes T, Bao T, Sabnis G, Brodie A. Aromatase, aromatase inhibitors, and breast cancer. *J Steroid Biochem Mol Biol* 2011;125:13–22.
- Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M, Coombes C, Snowdon C, Gnant M, Jakesz R, Kaufmann M, Boccardo F, Godwin J, Davies C, Peto R. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* 2010;28:509–18.
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 2011;378:771–84.
- van de Velde CJ, Rea D, Seynaeve C, Putter H, Hasenburger A, Vannetzel JM, Paridaens R, Markopoulos C, Hozumi Y, Hille ET, Kieback DG, Asmar L, Smeets J, Nortier JW, Hadji P, Bartlett JM, Jones SE. Adjuvant tamoxifen and exemestane in early breast cancer (TEAM): a randomised phase 3 trial. *Lancet* 2011;377:321–31.
- Regan MM, Neven P, Giobbie-Hurder A, Goldhirsch A, Ejlersen B, Mauriac L, Forbes JF, Smith I, Lang I, Wardley A, Rabaglio M, Price KN, Gelber RD, Coates AS, Thurlimann B; for the members of the BIG 1-98 Collaborative Group and the International Breast Cancer Study Group (IBCSG). Assessment of letrozole and tamoxifen alone and in sequence for postmenopausal women with steroid hormone receptor-positive breast cancer: the BIG 1-98 randomised clinical trial at 8.1 years median follow-up. *Lancet Oncol* 2011;12:1101–8.
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;365:1687–717.
- Fisher B, Constantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N, Other National Surgical Adjuvant Breast and Bowel Project Investigators. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;90:1371–88.
- Fisher B, Constantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, Atkins JN, Margolese RG, Runowicz CD, James JM, Ford LG, Wolmark N. Tamoxifen for the prevention of breast cancer: current status of the national surgical adjuvant breast and bowel project P-1 study. *J Natl Cancer Inst* 2005;97:1652–62.
- Jordan VC, Phelps E, Lindgren JU. Effects of antiestrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat* 1987;10:31–5.
- Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987;47:4020–4.
- Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res* 1990;50:4177–89.
- Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;48:812–5.
- Fornander T, Cedermark B, Mattsson A, Skloog L, Theve T, Askergren J, Rutqvist L, Glas U, Silfversward C, Somell A, Wilking N, Hjalmar ML. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet* 1989;ii:117–20.
- Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *J Am Med Assoc* 1999;281:2189–97.
- Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ, Cummings SR, CORE Investigators. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004;96:1751–61.
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER Jr, Wade JL 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N; National Surgical Adjuvant Breast and Bowel Project (NSABP). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *J Am Med Assoc* 2006;295:2727–41.
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL 3rd, Robidoux A, Margolese RG, James J, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N, National Surgical Adjuvant Breast and Bowel Project. Update of the national surgical adjuvant breast and bowel project study of tamoxifen and raloxifene (STAR) P-2 trial: preventing breast cancer. *Cancer Prev Res* 2010;3:696–706.
- Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. *CA: A Cancer J Clinicians* 2010;60:277–300.
- Jordan VC, Fritz NF, Lagan-Fahey S, Thompson M, Tormey DC. Alteration of endocrine parameters in premenopausal women with breast cancer during long-term adjuvant therapy with tamoxifen as the single agent. *J Natl Cancer Inst* 1991;83:1488–91.
- Murdtet TE, Schroth W, Bacchus-Gerybadze L, Winter S, Heinkele G, Simon W, Fasching PA, Fehm T; German Tamoxifen and AI Clinicians Group, Eichelbaum M, Schwab M, Brauch H. Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma. *Clin Pharmacol Ther* 2011;89:708–17.
- Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ. Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res* 1978;38:3352–64.

26. Allegra JC, Lippman ME. The effects of 17 β estradiol and tamoxifen on the ZR-75-1 human breast cancer cell line in defined medium. *Eur J Cancer* 1980;16:1007–15.
27. Lasfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. *J Natl Cancer Inst* 1978;61:967–78.
28. Kraus MH, Popescu NC, Amsburgh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J* 1987;6:605–10.
29. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, Brenner HJ. Establishment and characterization of a cell line of human breast carcinoma origin. *Eur J Cancer* 1979;15:659–70.
30. Murphy CS, Meisner LF, Wu SQ, Jordan VC. Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. *Eur J Cancer Clin Oncol* 1989;25:1777–88.
31. Chalbos D, Vignon F, Keydar I, Rochefort H. Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). *J Clin Endocrinol Metab* 1982;55:276–83.
32. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res* 1997;57:3071–8.
33. Soule MD, Vazquez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 1973;51:1409–13.
34. Brooks SC, Locke ER, Soule HD. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J Biol Chem* 1973;248:6251–3.
35. Lippman ME, Bolan G. Oestrogen-responsive human breast cancer in long-term tissue culture. *Nature* 1975;258:339–41.
36. Lippman M, Bolan G, Huff K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Res* 1976;36:4595–601.
37. Soule HD, McGrath CM. Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett* 1980;10:177–89.
38. Shafie SM. Estrogen and the growth of breast cancer: new evidence suggests indirect action. *Science* 1980;209:701–2.
39. Welshons WV, Wolf MF, Murphy CS, Jordan VC. Estrogenic activity of phenol red. *Mol Cell Endocrinol* 1988;57:169–78.
40. Bindal RD, Katzenellenbogen JA. Bis(4-hydroxyphenyl)[2-(phenoxy-sulfonyl)phenyl]methane: isolation and structure elucidation of a novel estrogen from commercial preparations of phenol red (phenolsulfonphthalein). *J Med Chem* 1988;31:1978–83.
41. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA* 1986;83:2496–500.
42. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* 1987;47:4355–60.
43. Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 β -estradiol. *J Natl Cancer Inst* 2001;93:1714–23.
44. Lewis JS, Meeke K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;97:1746–59.
45. Cho HS, NG PA, Katzenellenbogen BS. Differential regulation of gene expression by estrogen in estrogen growth-independent and -dependent MCF-7 human breast cancer cell sublines. *Mol Endocrinol* 1991;5:1323–30.
46. Welshons WV, Jordan VC. Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *Eur J Cancer Clin Oncol* 1987;23:1935–9.
47. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991;51:3867–73.
48. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;56:2321–30.
49. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* 1992;24:85–95.
50. Clarke R, Brunner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME, Dickson RB. Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both in vitro and in vivo. *Proc Natl Acad Sci* 1989;86:3649–53.
51. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* 1993;53:283–90.
52. Brunner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME, Clarke R. MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. *Cancer Res* 1993;53:3229–32.
53. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SA, Clarke R. MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. *Cancer Res* 1997;57:3486–93.
54. Bronzert DA, Greene GL, Lippman ME. Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY117018. *Endocrinology* 1985;117:1409–17.
55. Liu H, Lee ES, Gajdos C, Pearce ST, Chen B, Osipo C, Loweth J, McKian K, De Los Reyes A, Wing L, Jordan VC. Apoptotic action of 17 β -estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. *J Natl Cancer Inst* 2003;95:1586–97.
56. Balaburski GM, Dardes RC, Johnson M, Haddad B, Zhu F, Ross EA, Sengupta S, Klein-Szanto A, Liu H, Lee ES, Kim H, Jordan VC. Raloxifene-stimulated experimental breast cancer with the paradoxical actions of estrogen to promote or prevent tumor growth: a unifying concept in anti-hormone resistance. *Int J Oncol* 2010;37:387–98.
57. Liu H, Cheng D, Weichel AK, Osipo C, Wing LK, Chen B, Louis TE, Jordan VC. Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. *Int J Oncol* 2006;29:1237–46.
58. Masamura S, Santner SJ, Heitjan DF, Santen RJ. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab* 1995;80:2918–25.
59. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992;90:77–86.

60. Lewis JS, Osipo C, Meeke K, Jordan VC. Estrogen-induced apoptosis in a breast cancer model resistant to long-term estrogen withdrawal. *J Steroid Biochem Mol Biol* 2005;94:131–41.
61. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;55:2583–90.
62. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM, Jordan VC. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucleic Acids Res* 1996;24:962–9.
63. Vojtesek B, Lane DP. Regulation of p53 protein expression in human breast cancer cell lines. *J Cell Sci* 1993;105:607–12.
64. Murphy CS, Pink JJ, Jordan VC. Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Res* 1990;50:7285–92.
65. Pink JJ, Bilimoria MM, Assikis J, Jordan VC. Irreversible loss of the oestrogen receptor in T47D breast cancer cells following prolonged oestrogen deprivation. *Br J Cancer* 1996;74:1227–36.
66. Huber M, Bahr I, Kratzschmar JR, Becker A, Muller EC, Donner P, Pohlenz HD, Schneider MR, Sommer A. Comparison of proteomic and genomic analyses of the human breast cancer cell line T47D and the antiestrogen-resistant derivative T47D-r. *Mol Cell Proteomics* 2004;3:43–55.
67. Horwitz KB, Freidenberg GR. Growth inhibition and increase of insulin receptors in antiestrogen-resistant T47DCO human breast cancer cells by progestins: implications for endocrine therapies. *Cancer Res* 1985;45:167–73.
68. Graham ML 2nd, Krett NL, Miller LA, Leslie KK, Gordon DF, Wood WM, Wei LL, Horwitz KB. T47DCO cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res* 1990;50:6208–17.
69. van ben Berg HW, Lynch M, Martin J, Nelson J, Dickson GR, Crockard AD. Characterisation of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell line (ZR-75-9a1) and the ability of the resistant phenotype. *Br J Cancer* 1989;59:522–6.
70. Satyaswaroop PG, Zaino RJ, Mortel R. Human endometrial adenocarcinoma transplanted into nude mice: growth regulation by estradiol. *Science* 1983;219:58–60.
71. Zaino RJ, Satyaswaroop PG, Mortel R. Hormonal therapy of human endometrial adenocarcinoma in a nude mouse model. *Cancer Res* 1985;45:539–41.
72. Jordan VC, Gottardis MM, Satyaswaroop PG. Tamoxifen-stimulated growth of human endometrial carcinoma. *Ann N Y Acad Sci* 1991;622:439–46.
73. Jordan VC, Robinson SP. Species-specific pharmacology of antiestrogens: role of metabolism. *Fed Proc* 1987;46:1870–4.
74. Gottardis MM, Ricchio ME, Satyaswaroop PG, Jordan VC. Effect of steroidal and nonsteroidal antiestrogens on the growth of a tamoxifen-stimulated human endometrial carcinoma (EnCa101) in athymic mice. *Cancer Res* 1990;50:3189–92.
75. Addo S, Yates RA, Laight A. A phase I trial to assess the pharmacology of the new estrogen receptor antagonist fulvestrant on the endometrium in healthy postmenopausal volunteers. *Br J Cancer* 2002;87:1354–9.
76. Ilino Y, Wolf DM, Langan-Fahey SM, Johnson DA, Ricchio M, Thompson ME, Jordan VC. Reversible control of oestradiol-stimulated growth of MCF-7 tumours by tamoxifen in the athymic mouse. *Br J Cancer* 1991;64:1019–24.
77. Osborne CK, Hobbs K, Clark GM. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. *Cancer Res* 1985;45:584–90.
78. Gottardis MM, Robinson SP, Jordan VC. Estradiol-stimulated growth of MCF-7 tumors implanted in athymic mice: a model to study the tumorigenic action of tamoxifen. *J Steroid Biochem* 1988;30:311–4.
79. Osborne CK, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 1987;23:1189–96.
80. Gottardis MM, Jordan VC. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Res* 1988;48:5183–7.
81. Gottardis MM, Jiang SY, Jeng MH, Jordan VC. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res* 1989;49:4090–3.
82. Lee ES, Schafer JM, Yao K, England G, O'Regan RM, De Los Reyes A, Jordan VC. Cross-resistance of triphenylethylene-type antiestrogens but not ICI 182,780 in tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:4893–9.
83. O'Regan RM, Cisneros A, England GM, MacGregor JJ, Muenzner HD, Assikis VJ, Bilimoria MM, Piette M, Dragan YP, Pitot HC, Chatterton R, Jordan VC. Effects of the antiestrogens tamoxifen, toremifene, and ICI 182,780 on endometrial cancer growth. *J Natl Cancer Inst* 1998;90:1552–8.
84. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res* 1993;127:23–33.
85. Yao K, Lee ES, Bentrem DJ, England G, Schafer JJ, O'Regan RM, Jordan VC. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res* 2000;6:2028–36.
86. Schafer JM, Lee ES, O'Regan RM, Yao K, Jordan VC. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. *Clin Cancer Res* 2000;6:4373–80.
87. Schafer JM, Lee ES, Dardes RC, Bentrem D, O'Regan RM, De Los Reyes A, Jordan VC. Analysis of cross-resistance of the selective estrogen receptor modulators arzoxifene (LY353381) and LY117018 in tamoxifen-stimulated breast cancer xenografts. *Clin Cancer Res* 2001;7:2505–12.
88. Yue W, Zhou D, Chen S, Brodie A. A new nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene. *Cancer Res* 1994;54:5092–5.
89. Macedo LF, Sabnis GJ, Goloubeva OG, Brodie A. Combination of anastrozole with fulvestrant in the intratumoral aromatase xenograft model. *Cancer Res* 2008;68:3516–22.
90. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell* 2004;5:207–13.
91. Jordan VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death? *J Clin Oncol* 2008;26:3078–82.
92. Osborne CK, Phippen J, Jones SE, Parker LM, Ellis M, Come S, Gertler SZ, May JT, Burton G, Dimery I, Webster A, Morris C, Elledge R, Buzdar A. Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* 2002;20:3386–95.
93. Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, Kleeberg UR, Vergote I, Erikstein B, Webster

- A, Morris C. Fulvestrant, formerly ICI 182,780 is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* 2002;20:3396–403.
94. Di Leo A, Jerusalem G, Petruzella L, Torres R, Bondarenko IN, Khasanov R, Verhoeven D, Pedrini JL, Smirnova I, Lichinitser MR, Pendergrass K, Garnett S, Lindemann JP, Sapunar F, Martin M. Results of the CONFIRM phase III trial comparing fulvestrant 250 mg with fulvestrant 500 mg in postmenopausal women with estrogen receptor-positive advanced breast cancer. *J Clin Oncol* 2010;28:4594–600.
 95. Ellis MJ, Gao F, Dehdashti F, Jeffe DB, Marcom PK, Carey LA, Dickler MN, Silverman P, Fleming GF, Kommareddy A, Jamalabadi-Majidi S, Crowder R, Siegel BA. Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *J Am Med Assoc* 2009;302:774–80.
 96. Osipo C, Gajdos C, Liu H, Chen B, Jordan VC. Paradoxical action of fulvestrant in estradiol-induced regression of tamoxifen-stimulated breast cancer. *J Natl Cancer Inst* 2003;95:1597–608.
 97. Long BJ, Jelovac D, Thiantanawat A, Brodie AM. The effect of second-line antiestrogen therapy on breast tumor growth after first-line treatment with aromatase inhibitor letrozole: long-term studies using the intratumoral aromatase postmenopausal breast cancer model. *Clin Cancer Res* 2002;8:2378–88.
 98. Brodie A, Jelovac D, Long BJ. Predictions from a preclinical model: studies of aromatase inhibitors and antiestrogens. *Clin Cancer Res* 2003;9:455S–9S.
 99. Brodie A, Macedo L, Sabnis G. Aromatase resistance mechanisms in model systems in vivo. *J Steroid Biochem Mol Biol* 2010;118:283–7.
 100. Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CG, Nicolas E, Balagurunathan Y, Ross EA, Jordan VC. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci USA* 2011;108:18879–86.
 101. Wolf DM, Jordan VC. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. *Breast Cancer Res Treat* 1994;31:129–38.
 102. Levenson AS, Jordan VC. The key to the antiestrogenic mechanism of raloxifene is amino acid 351 (aspartate) in the estrogen receptor. *Cancer Res* 1998;58:1872–5.
 103. MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. *Cancer Res* 2000;60:5097–105.
 104. Liu H, Lee ES, Deb Los Reyes A, Zapf JW, Jordan VC. Silencing and reactivation of the selective estrogen receptor-estrogen receptor alpha complex. *Cancer Res* 2001;61:3632–9.
 105. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* 1994;54:2552–5.
 106. Prag G, Misra S, Jones EA, Ghirlando R, Davies BA, Horazdovsky BF, Hurley JH. Mechanism of ubiquitin recognition by the CUE domain of Vps9p. *Cell* 2003;113:609–20.
 107. Pan X, Zhou T, Tai YH, Wang C, Zhao J, Cao Y, Chen Y, Zhang PJ, Yu M, Zhen C, Mu R, Bai ZF, Li HY, Li AL, Liang B, Jian Z, Zhang WN, Man JH, Gao YF, Gong WL, Wei LX, Zhang XM. Elevated expression of CUEDC2 protein confers endocrine resistance in breast cancer. *Nat Med* 2011;17:708–14.
 108. Chumsri S, Sabnis GJ, Howes T, Brodie AM. Aromatase inhibitors and xenograft studies. *Steroids* 2011;76:730–5.
 109. Cormier EM, Wolf MF, Jordan VC. Decrease in estradiol-stimulated progesterone receptor production in MCF-7 cells by epidermal growth factor and possible clinical implication for paracrine-regulated breast cancer growth. *Cancer Res* 1989;49:576–80.
 110. Robinson SP, Jordan VC. The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: possible role in antiestrogen failure. *Eur J Cancer Clin Oncol* 1989;25:493–7.
 111. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev* 2008;29:217–33.
 112. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* 2011;62:233–47.
 113. Fox EM, Miller TW, Balko JM, Kuba MG, Sanchez V, Smith RA, Liu S, Gonzalez-Angulo AM, Mills GB, Ye F, Shyr Y, Manning HC, Buck E, Arteaga CL. A kinome-wide screen identifies the insulin/IGF-I receptor pathway as a mechanism of escape from hormone dependence in breast cancer. *Cancer Res* 2011;71:6773–84.
 114. van Agthoven T, van Agthoven TL, Portengen H, Foekens JA, Dorssers LC. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res* 1992;52:5082–8.
 115. van Agthoven T, van Agthoven TL, Dekker A, Foekens JA, Dorssers LC. Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. *Mol Endocrinol* 1994;8:1474–83.
 116. Cormier EM, Jordan VC. Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by estradiol or epidermal growth factor. *Eur J Cancer Clin Oncol* 1989;25:57–63.
 117. Turner N, Pearson A, Sharpe R, Lambros M, Geyer F, Lopez-Garcia MA, Natrajan R, Marchio C, Iorns E, Mackay A, Gillett C, Grigoriadis A, Tutt A, Reis-Filho JS, Ashworth A. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Cancer Res* 2010;70:2085–94.
 118. Treeck O, Wackwitz B, Haus U, Ortmann O. Effects of a combined treatment with mTOR inhibitor RAD001 and tamoxifen in vitro on growth and apoptosis of human cancer cells. *Gynecol Oncol* 2006;102:292–9.
 119. Abrahale T, Brodie A, Sabnis G, Macedo L, Tian C, Yue B, Serrero G. GP88 (PC-Cell Derived Growth Factor, progranulin) stimulates proliferation and confers letrozole resistance to aromatase overexpressing breast cancer cells. *BMC Cancer* 2011;11:231.
 120. Brodie A, Sabnis G. Adaptive changes result in activation of alternate signaling pathways and acquisition of resistance to aromatase inhibitors. *Clin Cancer Res* 2011;17:4208–13.
 121. Sabnis G, Brodie A. Adaptive changes results in activation of alternate signaling pathways and resistance to aromatase inhibitor resistance. *Mol Cell Endocrinol* 2011;340:142–7.
 122. Brodie A, Sabnis G, Macedo L. Xenograft models for aromatase inhibitor studies. *J Steroid Biochem Mol Biol* 2007;106:119–24.
 123. Macedo LF, Sabnis G, Brodie A. Preclinical modeling of endocrine response and resistance: focus on aromatase inhibitors. *Cancer* 2008;112:679–88.

124. Sabnis G, Brodie A. Understanding resistance to endocrine agents: molecular mechanisms and potential for intervention. *Clin Breast Cancer* 2010;10:E6–15.
125. Brodie A, Jelovac D, Sabnis G, Long B, Macedo L, Goloubeva O. Model systems: mechanisms involved in the loss of sensitivity to letrozole. *J Steroid Biochem Mol Biol* 2005;95:41–8.
126. Sabnis G, Goloubeva O, Jelovac D, Schayowitz A, Brodie A. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens. *Clin Cancer Res* 2007;13:2751–7.
127. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, Wong J, Allred DC, Clark GM, Schiff R. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003;95:353–61.
128. Lahusen T, Henke RT, Kagan BL, Wellstein A, Riegel AT. The role and regulation of the nuclear receptor co-activator AIB1 in breast cancer. *Breast Cancer Res Treat* 2009;116:225–37.
129. Hu ZZ, Kagan BL, Ariazi EA, Rosenthal DS, Zhang L, Li JV, Huang H, Wu C, Jordan VC, Riegel AT, Wellstein A. Proteomic analysis of pathways involved in estrogen-induced growth and apoptosis of breast cancer cells. *PLoS One* 2011;6:e20410.
130. Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. *Br Med J* 1944;2:393–8.
131. Ingle JN, Ahmann DL, Green SJ, Edmonson JH, Bisel HF, Kvols LK, Nichols WC, Creagan ET, Hahn RG, Rubin J, Frytak S. Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *N Engl J Med* 1981;304:16–21.
132. Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S, Women's Health Initiative Steering Committee. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *J Am Med Assoc* 2004;291:1701–12.
133. Nahta R, Esteva FJ. Bcl-2 antisense oligonucleotides: a potential novel strategy for the treatment of breast cancer. *Semin Oncol* 2003;30:143–9.
134. Song RX, Zhang Z, Mor G, Santen RJ. Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER(+) breast cancer cells. *Apoptosis* 2005;10:667–78.
135. Zhou H, Zhang Y, Fu Y, Chan L, Lee AS. Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). *J Biol Chem* 2011;286:25687–96.
136. Osipo C, Meeke K, Cheng D, Weichel A, Bertucci A, Liu H, Jordan VC. Role for HER2/neu and HER3 in fulvestrant-resistant breast cancer. *Int J Oncol* 2007;30:509–20.
137. Greene GL, Nolan C, Engler JP, Jensen EV. Monoclonal antibodies to human estrogen receptor. *Proc Natl Acad Sci USA* 1980;77:5115–9.
138. Greene GL, Fitch FW, Jensen EV. Monoclonal antibodies to estrophilin: probes for the study of estrogen receptors. *Proc Natl Acad Sci USA* 1980;77:157–61.
139. Poulsen HS, Ozzello L, King WJ, Greene GL. The use of monoclonal antibodies to estrogen receptors (ER) for immunoperoxidase detection of ER in paraffin sections of human breast cancer tissue. *J Histochem Cytochem* 1985;33:87–92.
140. DeSombre ER, Thorpe SM, Rose C, Blough RR, Andersen KW, Rasmussen BB, King WJ. Prognostic usefulness of estrogen receptor immunocytochemical assays for human breast cancer. *Cancer Res* 1986;46:4256s–64s.
141. Bevitt DJ, Milton ID, Piggot N, Henry L, Carter MJ, Toms GL, Lennard TW, Westley B, Angus B, Horne CH. New monoclonal antibodies to oestrogen and progesterone receptors effective for paraffin section immunohistochemistry. *J Pathol* 1997;183:228–32.
142. King WJ, Greene GL. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* 1984;307:745–7.
143. Ford CH, Al-Bader M, Al-Ayadhi B, Francis I. Reassessment of estrogen receptor expression in human breast cancer cell lines. *Anticancer Res* 2011;31:521–7.
144. Cao S, Hudnall SD, Kohen F, Lu LJ. Measurement of estrogen receptors in intact cells by flow cytometry. *Cytometry* 2000;41:109–14.
145. Girdler F, Browell DA, Cunliffe WJ, Shenton BK, Hemming JD, Scorer P, Young JR, Brotherick I. Use of the monoclonal antibody DAKO-ERbeta (8D5-1) to measure oestrogen receptor beta in breast cancer cells. *Cytometry* 2001;45:65–72.
146. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, Staub A, Jensen E, Scraze G, Waterfield M. Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci USA* 1985;82:7889–93.
147. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. *Science* 1986;231:1150–4.
148. Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 1986;320:134–9.
149. Catania C, Ascione G, Adamoli L, De Pas T, Medici M, Franceschelli L, Verri E, Magni E, Sanna G, Torrisi R, Goldhirsch A, Nolè F. Fulvestrant in heavily pre-treated patients with advanced breast cancer: results from a single compassionate use programme centre. *Breast Cancer Res Treat* 2007;106:97–103.
150. Jordan VC, Allen KE. Evaluation of the antitumor activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. *Eur J Cancer* 1980;16:239–51.
151. Brodie AM, Schwarzel WC, Shaikh AA, Brodie HJ. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology* 1977;100:1684–95.
152. Brodie AM, Marsh DA, Wu JT, Brodie HJ. Aromatase inhibitors and their use in controlling oestrogen-dependent processes. *J Steroid Biochem* 1979;11:107–12.
153. Brodie AM, Garrett WM, Hendrickson JR, Tsai-Morris CH. Effects of aromatase inhibitor 4-hydroxyandrostenedione and other compounds in the 7,12-dimethylbenz(a)anthracene-induced breast carcinoma model. *Cancer Res* 1982;42:3360s–4s.
154. Jordan VC, Fenuik L, Allen KE, Cotton RC, Richardson D, Walpole AL, Bowler J. Structural derivatives of tamoxifen and

- oestradiol 3-methyl ether as potential alkylating antioestrogens. *Eur J Cancer* 1981;17:193–200.
155. Bucourt R, Vignau M, Torelli V. New biospecific adsorbents for the purification of estradiol receptor. *J Biol Chem* 1978;253:8221–8.
 156. Wakeling AE, Bowler J. Steroidal pure antioestrogens. *J Endocrinol* 1987;112:R7–10.
 157. Johnston S, Phipps J Jr, Pivot X, Lichinitser M, Sadeghi S, Dieras V, Gomez HL, Romieu G, Manikhas A, Kennedy MJ, Press MF, Maltzman J, Florance A, O'Rourke L, Oliva C, Stein S, Pegram M. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. *J Clin Oncol* 2009;27:5538–46.
 158. Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, Schiff R, Gutierrez C, Migliaccio I, Anagnostou VK, Rimm DL, Magill P, Sellers M. Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study. *Clin Cancer Res* 2011;17:1147–59.
 159. Cristofanilli M, Valero V, Mangalik A, Royce M, Rabinowitz I, Arena FP, Kroener JF, Curcio E, Watkins C, Bacus S, Cora EM, Anderson E, Magill PJ. Phase II, randomized trial to compare anastrozole combined with gefitinib or placebo in postmenopausal women with hormone receptor-positive metastatic breast cancer. *Clin Cancer Res* 2010;16:1904–14.
 160. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. *J Clin Oncol* 2011;29:4452–61.
 161. Baselga J, Semiglazov V, van Dam P, Manikhas A, Bellet M, Mayordomo J, Campone M, Kubista E, Greil R, Bianchi G, Steinseifer J, Molloy B, Tokaji E, Gardner H, Phillips P, Stumm M, Lane HA, Dixon JM, Jonat W, Rugo HS. Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. *J Clin Oncol* 2009;27:2630–7.
 162. Novartis Pharmaceuticals. Everolimus in Combination With Exemestane in the Treatment of Postmenopausal Women With Estrogen Receptor Positive Locally Advanced or Metastatic Breast Cancer who are Refractory to Letrozole or Anastrozole (BOLERO-2). In: ClinicalTrials.gov [Internet]. Bethesda (MD). National Library of Medicine (US). 2000- [cited 2011 Nov 16]. Available from: <http://clinicaltrials.gov/show/NCT00863655> NLM Identifier: NCT00863655.
 163. Baselga J, Campone M, Piccart M, Burris HA, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, Beck JT, Ito Y, Yardley D, Deleu I, Perez A, Bachelot T, Vittori L, Xu Z, Mukhopadhyay P, Lebowitz D, Hortobagyi GN. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N Engl J Med* 2011 Dec 7. [Epub ahead of print].
 164. Sabnis G, Schayowitz A, Golubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. *Cancer Res* 2009;69:1416–28.
 165. Dodwell D, Coombes G, Bliss JM, Kilburn LS, Johnston S, SoFEA Trial Management Group. Combining fulvestrant (Faslodex) with continued oestrogen suppression in endocrine-sensitive advanced breast cancer: the SoFEA trial. *Clin Oncol (R Coll Radiol)* 2008;20:321–4.
 166. Swiss Group for Clinical Cancer Research. Trastuzumab and Letrozole in Treating Postmenopausal Women With Progressive Advanced Breast Cancer. In: ClinicalTrials.gov [Internet]. Bethesda (MD). National Library of Medicine (US). 2000- [cited 2011 Nov 15]. Available from: <http://clinicaltrials.gov/show/NCT00238290> NLM Identifier: NCT00238290.
 167. Koeberle D, Ruhstaller T, Jost L, Pagani O, Zaman K, von Moos R, Oehlschlegel C, Crowe S, Pilop C, Thuerlimann B, Swiss Group for Clinical Cancer Research (SAKK). Combination of trastuzumab and letrozole after resistance to sequential trastuzumab and aromatase inhibitor monotherapies in patients with estrogen receptor-positive, HER-2-positive advanced breast cancer: a proof-of-concept trial (SAKK 23/03). *Endocr Relat Cancer* 2011;18:257–64.
 168. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, Weiss H, Rimawi M, Schiff R. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res* 2008;68:826–33.
 169. Lonning PE, Taylor PD, Anker G, Iddon J, Wie L, Jorgensen LM, Mella O, Howell A. High-dose estrogen treatment in postmenopausal breast cancer patients heavily exposed to endocrine therapy. *Breast Cancer Res Treat* 2001;67:111–6.
 170. Sabnis GJ, Macedo LF, Golubeva O, Schayowitz A, Brodie AM. Stopping treatment can reverse acquired resistance to letrozole. *Cancer Res* 2008;68:4518–24.
 171. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a “new” biology of estrogen-induced apoptosis. *Cancer Prev Res (Phila)* 2011;4:633–7.
 172. LaCroix AZ, Chlebowski RT, Manson JE, Aragaki AK, Johnson KC, Martin L, Margolis KL, Stefanick ML, Brzyski R, Curb JD, Howard BV, Lewis CE, Wactawski-Wende J, WHI Investigators. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *J Am Med Assoc* 2011;305:1305–14.
 173. Heiser LM, Wang NJ, Talcott CL, Laderoute KR, Knapp M, Guan Y, Hu Z, Ziyad S, Weber BL, Laquerre S, Jackson JR, Wooster RF, Kuo WL, Gray JW, Spellman PT. Integrated analysis of breast cancer cell lines reveals unique signaling pathways. *Genome Biol* 2009;10:R31.
 174. Heiser LM, Sadanandam A, Kuo WL, Benz SC, Goldstein TC, Ng S, Gibb WJ, Wang NJ, Ziyad S, Tong F, Bayani N, Hu Z, Billig JJ, Dueregger A, Lewis S, Jakkula L, Korkola JE, Durinck S, Pepin F, Guan Y, Purdom E, Neuvial P, Bengtsson H, Wood KW, Smith PG, Vassilev LT, Hennessy BT, Greshock J, Bachman KE, Hardwicke MA, Park JW, Marton LJ, Wolf DM, Collisson EA, Neve RM, Mills GB, Speed TP, Feiler HS, Wooster RF, Haussler D, Stuart JM, Gray JW, Spellman PT. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci USA* 2011. [Epub ahead of print].
 175. Mendes-Pereira AM, Sims D, Dexter T, Fenwick K, Assiotis I, Kozarewa I, Mitsopoulos C, Hakas J, Zvelebil M, Lord CJ, Ashworth A. Genome-wide functional screen identifies a compendium of genes affecting sensitivity to tamoxifen. *Proc Natl Acad Sci USA* 2011 [Epub ahead of print].
 176. Liu X, Ory V, Chapman S, Yuan H, Timofeeva OA, Albanese C, Haddad B, Rhim JS, Dritschilo A, Riegel A, McBride A, Schlegel R. ROCK inhibitor and feeder cells induce the conditional immortalization of epithelial cells. *Am J Pathol* 2012;180:599–607.

POSITION STATEMENT

The 2012 Hormone Therapy Position Statement of The North American Menopause Society

Abstract

Objective: This position statement aimed to update the evidence-based position statement published by The North American Menopause Society (NAMS) in 2010 regarding recommendations for hormone therapy (HT) for postmenopausal women. This updated position statement further distinguishes the emerging differences in the therapeutic benefit-risk ratio between estrogen therapy (ET) and combined estrogen-progestogen therapy (EPT) at various ages and time intervals since menopause onset.

Methods: An Advisory Panel of expert clinicians and researchers in the field of women's health was enlisted to review the 2010 NAMS position statement, evaluate new evidence, and reach consensus on recommendations. The Panel's recommendations were reviewed and approved by the NAMS Board of Trustees as an official NAMS position statement.

Results: Current evidence supports the use of HT for perimenopausal and postmenopausal women when the balance of potential benefits and risks is favorable for the individual woman. This position statement reviews the effects of ET and EPT on many aspects of women's health and recognizes the greater safety profile associated with ET.

Conclusions: Recent data support the initiation of HT around the time of menopause to treat menopause-related symptoms and to prevent osteoporosis in women at high risk of fracture. The more favorable benefit-risk ratio for ET allows more flexibility in extending the duration of use compared with EPT, where the earlier appearance of increased breast cancer risk precludes a recommendation for use beyond 3 to 5 years.

Key Words: Bioidentical hormones – Breast cancer – Cardiovascular disease – Cognitive decline – Coronary heart disease – Dementia – Depression – Diabetes mellitus – Endometrial cancer – Estrogen – Estrogen-progestogen therapy – Estrogen therapy – Hormone therapy – Menopause – Mood – The North American Menopause Society – Osteoporosis – Ovarian cancer – Perimenopause – Postmenopause – Premature menopause – Premature ovarian insufficiency – Progestogen – Sexual function – Stroke – Total mortality – Urinary health – Quality of life – Vaginal atrophy – Vaginal health – Vasomotor symptoms – Venous thromboembolism – Women's Health Initiative.

The intent of The North American Menopause Society (NAMS) 2012 Hormone Therapy Position Statement is to clarify the benefit-risk ratio of estrogen therapy (ET) versus estrogen-progestogen therapy (EPT) for both treatment of menopause-related symptoms and disease prevention at various time intervals since menopause. The availability of long-term data related to the effects of hormone therapy (HT) both during and after use of HT prompted the

NAMS Board of Trustees to update its position statement. NAMS convened a seventh Advisory Panel to provide recommendations. The Panel's recommendations were reviewed and approved by the 2011-2012 NAMS Board of Trustees.

The term HT is used to encompass both ET and EPT when outcomes are not specific to one or the other treatment.

These statements do not represent codified practice standards as defined by regulating bodies and insurance agencies.

Received January 17, 2012; revised and accepted January 17, 2012.

This position statement was developed by The North American Menopause Society (NAMS) 2012 Hormone Therapy Position Statement Advisory Panel consisting of representatives of the NAMS Board of Trustees and other experts in women's health: Margery L.S. Gass, MD, NCMP (Co-Chair); JoAnn E. Manson, MD, DrPH, NCMP (Co-Chair); Felicia Cosman, MD; Francine Grodstein, ScD; V. Craig Jordan, OBE, PhD, DSc, FMedSci; Richard H. Karas, MD, PhD; Andrew M. Kaunitz, MD; Pauline M. Maki, PhD; Peter J. Schmidt, MD; Jan L. Shifren, MD, NCMP; Cynthia A. Stuenkel, MD, NCMP; and Wulf H. Utian, MD, PhD, DSc(Med), NCMP. The Board of Trustees approved the position statement on January 15, 2012.

Address correspondence to: The North American Menopause Society, 5900 Landerbrook Drive, Suite 390, Mayfield Heights, OH 44124. E-mail: info@menopause.org. Web site: www.menopause.org

METHODS

An Advisory Panel of clinicians and researchers expert in the field of women's health was enlisted to review the previous position statement of July 2010 (available at <http://www.menopause.org/PSHT10.pdf>), evaluate the literature published subsequently, and conduct an evidence-based analysis with the goal of reaching consensus on recommendations.

NAMS acknowledges that no single trial data can be extrapolated to all women. However, because the Women's Health Initiative (WHI) is, for some outcomes, the only large long-term randomized controlled trial (RCT) of postmenopausal women using HT, these findings were given prominent

consideration among all the studies reviewed in the development of this position statement. Nonetheless, the WHI hormone trials had several characteristics that limit generalizing the findings to all postmenopausal women. These include the use of only one route of administration (oral), only one formulation of estrogen (conjugated estrogens [CEs]), and only one progestogen (medroxyprogesterone acetate). Unlike most HT studies that focused on symptomatic, recently postmenopausal women, the WHI enrolled generally healthy postmenopausal women aged 50 to 79 years in a prevention trial. These parameters should be taken into consideration when applying the WHI findings to clinical practice as should be the findings from observational studies with their known limitations. In general, the panel gave more weight to RCTs.

BENEFITS AND RISKS OF HORMONE THERAPY

Vasomotor symptoms

ET with or without a progestogen is the most effective treatment of menopause-related vasomotor symptoms and their potential consequences, such as diminished sleep quality, irritability, difficulty concentrating, and subsequently reduced quality of life (QOL).^{1,2} Treatment of moderate to severe vasomotor symptoms remains the primary indication for HT. Almost all systemic HT products except for the ultralow-dose estradiol transdermal patch (approved for the prevention of osteoporosis) have government approval for this indication.³ Progestogen alone also reduces vasomotor symptoms but not as effectively as estrogen does.⁴

Vaginal symptoms

ET is the most effective treatment of moderate to severe symptoms of vulvar and vaginal atrophy (eg, vaginal dryness, dyspareunia, and atrophic vaginitis).⁵ Many systemic HT products and all local vaginal ET products have government approval for treating symptomatic vaginal atrophy. Some low-dose systemic regimens may be inadequate for the relief of vaginal symptoms and may require the addition of low-dose local ET to achieve the desired results. When ET is considered solely for treatment of vaginal atrophy, local vaginal ET is advised. Lower doses of vaginal ET than previously used, with less frequent administration, often yield satisfactory results.⁶

A progestogen is generally not indicated when ET at the recommended low doses is administered locally for vaginal atrophy, although clinical trial data supporting endometrial safety beyond 1 year are lacking.⁷ Because endometrial hyperplasia increases with increasing dose and duration of estrogen exposure, thorough evaluation of any uterine bleeding in women using low-dose local ET is advised.

Sexual function

A significant effect of ET on sexual interest, arousal, and orgasmic response independent from its role in treating menopausal symptoms is not supported by current evidence.⁸ Low-dose local ET may improve sexual satisfaction by improving lubrication and increasing blood flow and sensa-

tion in vaginal tissues. In an analysis of the persistence of sexual activity in the WHI, HT was not correlated with longer persistence of sexual activity.⁹ HT is not recommended as the sole treatment of other problems of sexual function, including diminished libido.¹⁰

Urinary tract health

Local ET may benefit some women with overactive bladder.¹¹ One RCT found that an estradiol ring had a clinical benefit equivalent to that of oxybutynin among women with overactive bladder.¹² Systemic HT may worsen or provoke stress incontinence.¹³⁻¹⁵ Ultralow-dose transdermal estradiol therapy neither increased nor decreased incontinence.¹⁶ A large RCT reported an increased risk of kidney stones with HT.¹⁷

Two studies reported a decreased risk of recurrent urinary tract infection through the use of intravaginal estrogen.^{18,19} Only ET administered by the vaginal route has been shown to be effective for this purpose. No HT product has government approval for any urinary health indication.

Quality of life

Although no HT product has government approval for enhancing QOL, use of HT can result in an improvement in health-related QOL (HQOL) in symptomatic women through the alleviation of symptoms.^{1,2,20} There is no clear evidence that HT improves HQOL in asymptomatic women.²⁰⁻²³ With regard to physical functioning as a measure of HQOL, data from the WHI found no benefit of HT in women 65 years or older when measured for grip strength, chair standing, and walking.²⁴

Osteoporosis

There is RCT evidence that standard-dose HT reduces postmenopausal osteoporotic fractures, including hip, spine, and all nonspine fractures, even in women without osteoporosis.^{25,26} Low doses are effective in maintaining or improving bone mineral density. No HT product currently has government approval for the treatment of osteoporosis. Many systemic HT products, however, have government approval for the prevention of postmenopausal osteoporosis.

When alternate osteoporosis therapies are not appropriate or cause adverse effects, the extended use of HT is an option for women who are at high risk of osteoporotic fracture. There is no evidence that HT stops working with long-term treatment; however, the benefits of HT on bone mass and fracture reduction dissipate quickly after the discontinuation of treatment,^{27,28} necessitating a transition to a different osteoporosis treatment (or prevention strategy) to preserve bone mass. Within a few years of the discontinuation of ET in the WHI, the cumulative incidence of hip fracture was the same in the ET and placebo groups.²⁸

Unless there is a contraindication, women experiencing an early menopause who require prevention of bone loss are probably best served by the administration of HT or oral contraceptives, rather than other bone-specific treatments, until they reach the normal age of menopause at which time treatment may be reassessed. The presumed increased risk of fracture in

older women who had an early menopause, however, was not substantiated in a recent report from the Study of Osteoporotic Fractures.²⁹ Women older than 65 years with a history of early menopause and no HT use did not sustain more fractures than did the group who had menopause at the average age. Removal of both ovaries at the time of hysterectomy compared with ovarian conservation was similarly found not to increase the subsequent rate of hip fracture.³⁰

Cardiovascular effects

The cardiovascular effects discussed are coronary heart disease (CHD), carotid intima media thickness, coronary artery calcium, stroke, and venous thromboembolism (VTE).

Coronary heart disease

Most observational studies (primarily composed of women who began HT around the time of menopause) support the potential benefits of systemic HT in reducing the risk of CHD.³¹ Most RCTs do not.^{31,32} However, it is understood that the characteristics of women participating in observational studies are markedly different from those of many women enrolled in RCTs designed to evaluate the cardiovascular effects of HT.³³⁻³⁵ These demographic and biologic differences can influence baseline cardiovascular risks and may modify the overall observed effects of HT on cardiovascular risk. In the WHI clinical trials, overall CHD risk was estimated to be increased by eight cases per 10,000 women per year in the EPT arm; in the ET arm, overall CHD risk was estimated to be decreased by three cases per 10,000 women per year³⁶ (see "Dose and route of administration").

Timing of initiation. Secondary analyses of the WHI data indicate that the disparity in findings between observational studies and RCTs is related partly to the timing of initiation of HT in relation to age and proximity to menopause.^{36,37} Most participants in the observational studies of CHD risk were younger than 55 years at the time HT was initiated and within 2 to 3 years of menopause. On the other hand, women enrolled, to date in RCTs with clinical cardiovascular endpoints have an age of 63 to 64 years and are more than 10 years beyond menopause. When analyzed by age and time since menopause at initiation of HT, the ET arm of the WHI³⁸ is in general agreement with observational studies³⁹ suggesting that ET may reduce CHD risk (coronary revascularization and composite outcomes including myocardial infarction [MI] and coronary death) when initiated in younger and more recently postmenopausal women without a uterus.³⁶ These findings for ET were even stronger with extended follow-up of the cohort and inclusion of 4 years after stopping. For women ages 50 to 59 years, the hazard ratio (HR) for CHD was 0.59 (95% CI, 0.38-0.90); for total MI, it was 0.54 (95% CI, 0.34-0.85; *P* for interaction by age = 0.05 and 0.007, respectively).²⁸

Combined data incorporating both the ET and EPT trials of the WHI show a statistical trend of an HT effect relative to placebo on CHD by time since menopause, indicating that the women who initiate HT more than 10 years beyond menopause are at increased risk for CHD, and those women who initiate HT

within 10 years of menopause tend to have a lower risk of CHD.³⁶ However, statistical modeling of the combined WHI data, including data from the WHI observational studies, did not find that CHD risks varied by the timing of HT initiation.^{36,40}

Coronary artery calcium. Some observational studies,^{41,42} but not all,⁴³ suggest that long-term HT is associated with less accumulation of coronary artery calcium, which is strongly correlated with atheromatous plaque burden and future risk of clinical CHD events. In an ancillary substudy of younger women (<60 y) in the WHI ET trial, after an average of 7 years of treatment, women who had been randomized to ET had lower levels of coronary artery calcium than did those randomized to placebo.⁴⁴ Although the effect in older women was not evaluated, these findings suggest that ET initiated by recently postmenopausal women may slow the development of calcified atherosclerotic plaque.

Carotid intima media thickness. Observational studies⁴⁵⁻⁴⁷ demonstrate less accumulation of carotid plaque as measured through ultrasound in women taking HT. Two RCTs reported contradictory findings with regard to carotid plaque.^{48,49}

Stroke

The WHI EPT and ET trials demonstrated an increased risk of ischemic stroke and no effect on the risk of hemorrhagic stroke.^{50,51} In these trials, when the entire cohort was analyzed, there were eight additional strokes per 10,000 women per year of EPT and 11 additional strokes per 10,000 women per year of ET. In recent analyses that combined results from the WHI EPT and ET trials, HT in younger women (ages 50-59 y) at study entry had no significant effect on risk of stroke (relative risk [RR], 1.13; 95% CI, 0.73-1.76).^{36,40}

Although stroke was not increased in the group ages 50 to 59 years in the combined analysis of the WHI, it was almost doubled in the ET group less than 10 years since menopause. This apparent contradiction in the data is hard to explain but may be caused by relatively few events and the difficulty in accurately timing the onset of menopause in the ET group. In both the ET and EPT trials, excess stroke risk dissipated rapidly after discontinuation of HT.^{27,28}

In women randomized in the WHI within 5 years of menopause, there were three additional strokes per 10,000 women per year of EPT, which is not statistically significant.³⁶ The excess risk of stroke in this age group observed in the WHI studies would fall into the rare-risk category. Stroke risk was not significantly increased in the Heart and Estrogen/Progestin Replacement Study⁵² and the Women's Estrogen for Stroke Trial secondary prevention trials.⁵³ The Women's International Study of long Duration Oestrogen after Menopause RCT found no excess risk of stroke in EPT users compared with women on placebo in 1 year.⁵⁴

The results of observational studies on the risk of stroke with HT have been inconsistent. Several studies (including the Nurses' Health Study [NHS], the largest and longest prospective cohort study of women's health) indicated an increased risk of ischemic stroke consistent with the findings from the WHI,⁵⁵ whereas other studies showed no effect on

stroke risk.⁵⁶⁻⁵⁸ In the NHS, among women ages 50 to 59 years, the RR of stroke for current EPT users was not significantly elevated (RR, 1.34; 95% CI, 0.84-2.13), but it was significantly increased for current users of ET among women ages 50 to 59 years (RR, 1.58; 95% CI, 1.06-2.37).⁵⁵ The lowest dose of estrogen (eg, 0.3 mg CE) was not associated with an increased risk in the NHS, although this was based on the relatively few women who were taking that dose (see “Dose and route of administration”).

Venous thromboembolism

Data from both observational studies and RCTs consistently demonstrate an increased risk of VTE with oral HT.^{59,60} In the WHI trials, when the entire cohort was analyzed, there were 18 additional VTEs per 10,000 women per year of EPT⁶⁰ and 7 additional VTEs per 10,000 women per year of ET.⁶¹ VTE risk in RCTs emerges soon after HT initiation (ie, during the first 1-2 y), and the magnitude of the excess risk seems to decrease somewhat in time. In the WHI trials, the absolute excess VTE risk associated with either EPT or ET was lower in women who started HT before age 60 years than in older women who initiated HT after age 60 years. In women ages 50 to 59 years who were randomized to HT,⁶¹ there were 11 additional VTEs per 10,000 women per year of EPT and 4 additional VTEs per 10,000 women per year of ET. These risks fall into the rare-risk category. The baseline risk of VTE also increased relative to body mass index (BMI). For obese women (BMI, >30 kg/m²), the baseline risk was almost threefold greater. At any BMI, the risk of VTE doubled with HT and returned to baseline soon after HT discontinuation.^{27,28}

Women with a previous history of VTE, obese women, or women who possess a factor V Leiden mutation are at increased risk of VTE with HT use.^{60,62,63} There are limited observational data suggesting lower risks of VTE with transdermal than with oral ET,⁶⁴⁻⁶⁶ but there are no comparative RCT data on this subject. Lower doses of oral ET may also confer less VTE risk than higher doses, but no comparative RCT data are available to confirm this assumption. Studies that have evaluated the contribution of various progestogens to clotting suggest that norepregnanes may be more thrombogenic.^{67,68}

HT is currently not recommended for coronary protection in women of any age. Initiation of HT by women ages 50 to 59 years or by those within 10 years of menopause to treat typical menopausal symptoms does not seem to increase the risk of CHD events. There is emerging evidence that the initiation of ET in early postmenopause may reduce coronary artery disease and CHD risk. Two ongoing studies of early HT intervention may provide further information on this topic: the Early versus Late Intervention Trial with Estradiol and the Kronos Early Estrogen Prevention Study.

Diabetes mellitus

Large RCTs demonstrate that HT reduces the diagnosis of new onset type 2 diabetes mellitus (T2DM), although no HT product has government approval to prevent T2DM. Women who received active treatment in the WHI EPT arm had a

statistically significant 21% reduction (HR, 0.79; 95% CI, 0.67-0.93) in the incidence of T2DM requiring treatment, which indicates 15 fewer cases per 10,000 women per year of therapy.⁶⁹ A similar statistically significant risk reduction was also noted in the Heart and Estrogen/Progestin Replacement Study trial (HR, 0.65; 95% CI, 0.48-0.89).⁷⁰ In the WHI ET trial, there was a 12% reduction (HR, 0.88; 95% CI, 0.77-1.01) in incident T2DM or 14 fewer cases per 10,000 women per year of ET.⁷¹ Unfortunately, none of these trials included an oral glucose tolerance test to evaluate postchallenge glucose levels. In the Postmenopausal Estrogen and Progestin Intervention trial, fasting glucose levels were reduced in women assigned to HT; however, 2-hour postchallenge glucose levels, which may be associated with CHD risk, were elevated.⁷² There is inadequate evidence to recommend HT for the sole or primary indication of the prevention of T2DM in perimenopausal or postmenopausal women.

Endometrial cancer

Unopposed systemic ET in postmenopausal women with an intact uterus is associated with increased endometrial cancer risk related to the ET dose and duration of use. A meta-analysis reported a summary RR of 2.3 (95% CI, 2.1-2.5) overall and an RR of 9.5 if used for more than 10 years.⁷³ This increased risk persisted for several years after ET discontinuation. To negate this increased risk, adequate concomitant progestogen is recommended for women with an intact uterus when using systemic ET (see “Progestogen indication”). In general, HT is not recommended in women with a history of endometrial cancer. Progestogen alone could be considered for the management of vasomotor symptoms but no long-term data are available.

Breast cancer

Estrogen-progestogen therapy

Diagnosis of breast cancer increases with EPT use beyond 3 to 5 years.⁷⁴ In the WHI overall, this increased risk, in absolute terms, was eight additional breast cancers per 10,000 women using EPT for 5 or more years. Studies have not clarified whether the risk differs between continuous and sequential use of progestogen, with observational studies suggesting that risk may be greater with continuous use of progestogen. It is also not clear whether there is a class effect with progestogens or whether the specific agent used influences the degree of breast cancer risk. Data from a large observational study suggest that EPT with micronized progesterone carries a low risk of breast cancer with short-term use but carries an increased risk of breast cancer with all EPT formulations with long-term use.⁷⁵

EPT and, to a lesser extent, ET increase breast cell proliferation, breast pain, and mammographic density, and EPT may impede the diagnostic interpretation of mammograms, therein delaying the diagnosis of breast cancer.^{74,76} Evolving but not conclusive evidence suggests that the increased risk of breast cancer with EPT may be a result of the promotion of

preexisting cancers that are too small to be diagnosed by imaging studies or clinical examination. Some of these small cancers may never progress without the stimulation of HT. Long-term follow-up found that the risk of new diagnosis of breast cancer dissipated in the 3 years after cessation of EPT.⁷⁷ However, the follow-up also revealed that breast cancer mortality was increased in EPT users in the WHI who were followed for 11 years after study initiation. The breast cancer death rates with EPT were two additional deaths per 10,000 women per year attributed to breast cancer and two additional deaths per 10,000 women per year attributed to all-cause mortality.⁷⁸

In the WHI, the initial reports suggested that the increase in breast cancer risk was limited to those who had used EPT before enrollment.⁷⁹ Because most women initiate EPT shortly after menopause, a reanalysis of the data examined the effect of a “gap time” (duration of time between onset of menopause and start of EPT) on breast cancer risk. In a combined analysis of the WHI observational study and the EPT clinical trial, those starting EPT shortly after menopause had an HR of 2.75 for breast cancer with more than 5 years of use, whereas those with a gap time of greater than 5 years did not.⁸⁰ A detailed secondary analysis reported that women who experienced a hiatus in their exposure to hormones before randomization to EPT were found to have a delayed increase in breast cancer compared with previous EPT users.⁸¹ The French E3N (a prospective cohort study of French women that examined the potential relationship between premenopausal and postmenopausal breast cancer occurrence) also reported a greater risk of breast cancer in those women with a short (<3 y) as opposed to those with a long gap time.⁷⁵ The Million Women Study (MWS) investigators reported an increased risk in women initiating HT shortly after menopause.⁸²

These data on breast cancer (potentially more harm with early postmenopausal HT use) are in contrast with the findings on CHD, stroke, VTE, and all-cause mortality that suggest greater safety in younger women closer to menopause. For all outcomes, the absolute risk of events in younger women is lower than that for older women.

Estrogen therapy

Women in the ET arm of the WHI demonstrated no increase in risk of breast cancer after an average of 7.1 years of use, with six fewer cases of invasive breast cancer per 10,000 women per year of ET use, which is not statistically significant.⁷⁶ The decrease in risk was observed in all three age groups studied (ages 50-59, 60-69, and 70-79 y). Other findings in the ET group included a reduction in ductal carcinomas (HR, 0.71; 95% CI, 0.52-0.99).⁷⁶ In analyses based on extended follow-up of the WHI ET trial, including after stopping, the HR for breast cancer was 0.77 (95% CI, 0.62-0.95).²⁸ However, in women assigned to CE who developed invasive breast cancer, fewer breast cancers presented with localized disease (HR, 0.69; 95% CI, 0.51-0.95), and tumors were larger and more likely to be node positive compared with those in women assigned to placebo.⁷⁶

The hypothesis for the decreased incidence of breast cancer with use of CE in the WHI is the apoptotic effect that estrogen has on breast cancer cells in a low-estrogen environment. Although the use of CE in the WHI did not show an age-related difference in the reduction of breast cancer, all laboratory evidence suggests that the longer breast cancer cells are estrogen-deprived, the more probable that physiologic estrogen will have a tumoricidal effect.⁸³

The decreased risk of breast cancer as seen in the ET arm of the WHI was not observed in the MWS.⁸² The RR for breast cancer in the MWS was increased in women who started ET within 5 years after menopause, with an absolute increased risk of 13 cases per 10,000 women per year.⁸² Whether the difference between these findings and the WHI ET arm reflects differences in the timing of ET initiation, the types of ET, study populations, increased mammographic surveillance of women using HT, or other factors not controlled for in an observational study has not been determined.

When ET was extended beyond 15 years in the NHS, breast cancer risk increased.⁸⁴⁻⁸⁶ A large meta-analysis of 67,370 women in observational studies found no increased risk with less than 5 years of ET use and RRs of 1.31 for 5 to 9 years of use, 1.24 for 10 to 14 years of use, and 1.56 for more than 15 years of use.⁸⁷ The possibility of differences in mammographic surveillance for breast cancer in users and nonusers of HT in observational studies cannot be excluded.

HT after breast cancer

Controversy surrounds the use of HT in survivors of breast cancer. Some observational studies suggest that HT use may not increase the risk of recurrent breast cancer.⁸⁸⁻⁹⁴ These reports have been questioned because of the potential bias from the selection of women at lower risk of recurrence for HT use. An RCT of HT use in women with a history of breast cancer and bothersome vasomotor symptoms was terminated early, after 2 years of follow-up, when significantly more new breast cancer events were diagnosed in women randomized to HT.⁹⁵ These data would indicate that HT use in breast cancer survivors may be associated with an increased risk of recurrence.

Ovarian cancer

Published data on the role of HT and risk of ovarian cancer are conflicting. Some studies did not find an association.^{96,97} There is a relatively large volume of observational trial data that points to an association between HT and increased ovarian cancer risk, particularly with long-term use.⁹⁸⁻¹⁰⁹ In the National Institutes of Health American Association of Retired Persons Diet and Health Cohort, no elevated risk of ovarian cancer was seen with less than 10 years of ET use, but a significantly increased risk was seen after 10 years.¹⁰⁷ One meta-analysis reported an increase in annual ovarian cancer risk for EPT of 1.11-fold (95% CI, 1.02-1.21), and a 1.28-fold (95% CI, 1.18-1.40) increase was reported for ET.¹¹⁰ A second meta-analysis reported RRs of 1.24 (95% CI, 1.15-1.34) for cohort studies and 1.19 (95% CI, 1.02-1.40) for case-control studies with use of any HT.¹¹¹ The use of HT for less than

5 years was associated with a significant RR of 1.03, whereas use for more than 10 years was associated with an RR of 1.21 ($P < 0.05$ for both RRs). ET was associated with a higher risk of ovarian cancer than EPT.

In the WHI, the only RCT to date to study ovarian cancer, EPT was not associated with a statistically significant increase in ovarian cancer after a mean of 5.6 years of use.¹¹² There were 4.2 cases per 10,000 for HT users and 2.7 cases per 10,000 per year for the placebo group.

The association between ovarian cancer and EPT use beyond 5 years would fall into the rare- or very rare-risk category. Women at increased risk of ovarian cancer (eg, those with a family history or a *BRCA* mutation) should be counseled about this potential association.

Lung cancer

In a post hoc analysis of the EPT arm of the WHI that included data from a mean of 7.1 years of intervention plus approximately 1 year of postintervention follow-up (total mean years of data, 7.9), the incidence of non-small-cell lung cancer (which accounts for about 80% of lung cancer) was not significantly increased (HR, 1.28; 95% CI, 0.94-1.73; $P = 0.12$), but the number of lung cancer deaths (from non-small-cell lung cancer) increased (HR, 1.87; 95% CI, 1.22-2.88; $P = 0.004$), and the number of poorly differentiated and metastatic tumors increased in the treatment group (HR 1.87; 95% CI, 1.22-2.88; $P = 0.004$).⁷⁷ The cases were essentially limited to past and current smokers and to women older than 60 years. The absolute rates of death from non-small-cell lung cancer were small: nine per 10,000 per year on EPT and five per 10,000 on placebo. Because the WHI was not designed to assess lung cancer and chest imaging was not part of the study protocol, the findings are preliminary and require validation in further studies.

In the WHI ET trial, no increase in lung cancer incidence or mortality was observed in the treatment compared with the placebo group.¹¹³ There was no significant treatment effect related to age. Mortality from lung cancer was increased in current smokers in both treatment and placebo groups compared with nonsmokers and former smokers.

Reports from observational trials are mixed.¹¹⁴⁻¹²² One large observational study reported an increase in incident lung cancer associated with increasing duration of EPT use (50% increase after 10 y of therapy); there was no association with duration of ET use.¹²³ One meta-analysis reported an increased risk of adenocarcinoma of the lung.¹²⁴ Another meta-analysis reported a possible protective effect against lung cancer for users of HT with the exception of current smokers.¹²⁵

These findings underscore the need to encourage the cessation of smoking and possibly to increase surveillance in older smokers who are current or past users of EPT.

Mood and depression

For postmenopausal women without clinical depression, evidence is mixed concerning the effects of HT on mood.

Several small short-term trials among middle-aged women with vasomotor symptoms suggested that HT improves mood, whereas other trial results showed no change. Progestogens in EPT may worsen mood in some women, possibly in those with a history of premenstrual syndrome, premenstrual depressive disorder, or clinical depression.

Only a few RCTs have examined the effects of HT in middle-aged or older women who have depression. One small RCT involving depressed perimenopausal and postmenopausal women found no short-term benefit from ET, but post hoc analyses revealed that higher estradiol levels were associated with decreased depressive symptoms in perimenopausal women but not postmenopausal women.¹²⁶ Two small RCTs support the antidepressant efficacy of short-term ET in depressed perimenopausal women,^{127,128} whereas one RCT failed to demonstrate the antidepressant efficacy of ET in depressed women who were 5 to 10 years into postmenopause.¹²⁹ It is controversial whether ET might, in some circumstances, augment the antidepressant effects of selective serotonin reuptake inhibitors.^{130,131}

Although HT might have a positive effect on mood and behavior, HT is not an antidepressant and should not be considered as such. Evidence is insufficient to support HT use in the treatment of depression.

Cognitive aging and dementia

Very small clinical trials support the use of ET for cognitive benefits when initiated immediately after surgical menopause.^{132,133} To date, clinical trials of ET have demonstrated no substantial effect on episodic memory or executive function at the time of menopause.¹³⁴ Reports from the longitudinal Study of Women's Health Across the Nation suggest that natural menopause has a significant but small effect on some aspects of cognitive function that may be time limited. This effect is not explained by menopausal symptoms.^{135,136} Recent literature suggests a transient negative effect of the menopausal transition on cognition, but it is a negligible long-term effect.^{134,135}

The NHS found no benefit on cognitive function from long-term use of HT among women who had started HT in early menopause; rather, there was a suggestion of a more rapid cognitive decline among HT users.¹³⁷ Conversely, in the Study of Women's Health Across the Nation, women who initiated hormones (oral contraceptives or HT) after enrollment but before their final menstrual period and then discontinued the hormones had a beneficial cognitive effect, whereas women who initiated hormones after the final menstrual period had a detrimental effect on cognitive performance.¹³⁵

For postmenopausal women older than 65 years, findings from several large well-designed clinical trials indicate that HT does not improve memory or other cognitive abilities and that EPT is harmful for memory.¹³⁸⁻¹⁴⁰ The WHI Memory Study of women aged 65 to 79 years reported an increase in dementia incidence with HT use.¹⁴¹ The estimate of dementia cases attributed to HT was 12 per 10,000 persons per year of ET use and 23 per 10,000 persons per year of EPT use. The

effect was not statistically significant for ET but was for EPT and the combined ET and EPT groups.¹⁴¹

Evidence from the WHI Study of Cognitive Aging, an ancillary study of WHI and WHI Memory Study that enrolled women aged 66 years or older, indicated a worsening of verbal memory but a trend toward a positive effect on figural memory among women using EPT compared with those using placebo.¹⁴⁰ There are currently no placebo-controlled trial data comparing the effects of different progestogens on memory or dementia in younger or older postmenopausal women. Overall, the RCTs of ET demonstrate no adverse impact on memory. The WHI Study of Cognitive Aging found neither benefit nor persistent negative impact of HT on memory during a 2.7-year interval.¹⁴²

A number of observational studies have reported associations between HT and reduced risk of developing Alzheimer disease (AD).¹⁴³ HT exposure in observational studies is more likely to involve ET use by younger women closer to menopause, suggesting an early window during which HT use might reduce AD risk. However, recall bias and the healthy-user bias may account for protective associations in the observational studies. Similarly, an increased risk of dementia observed with early oophorectomy, countered by use of estrogen until age 50 years,¹⁴⁴ may be at least partially caused by demographic differences between groups.¹⁴⁵ HT exposure in observational studies is also more likely to involve women on ET rather than EPT. For women with AD, limited clinical results suggest that ET has no substantial effect.

In summary, available data do not adequately address whether HT used soon after menopause increases or decreases the rate of cognitive decline or later dementia risk. In the absence of more definitive findings, HT cannot be recommended at any age for preventing or treating cognitive aging or dementia.

Premature menopause and primary ovarian insufficiency

Women experiencing premature menopause (age ≤ 40 y) or primary ovarian insufficiency (POI) are medically a distinctly different group from women who reach menopause at the median age of 51.3 years. Premature menopause and POI are associated with a lower risk of breast cancer and earlier onset of estrogen-related bone loss. Other conditions that have been associated with premature menopause, such as CHD and Parkinson disease, may be the result of other factors responsible for both premature menopause and the specific condition. For example, mutations found in the gene encoding mitochondrial DNA polymerase gamma have been reported to be associated with both premature menopause and Parkinson disease.¹⁴⁶

Some observational reports suggest an increased risk of CHD with early natural or surgical menopause in the absence of HT and a reduced risk when HT is administered.¹⁴⁷ Analysis of the Framingham data revealed that women who had an earlier menopause also had more CHD risk factors.¹⁴⁸ The authors concluded that CHD risk factors may cause earlier menopause and not the converse. Both a history of heart disease and smoking have been associated with earlier meno-

pause.¹⁴⁹ Another extensive analysis of three birth cohorts from three different countries concluded that there is no change in the rate of increase in CHD mortality at menopause. The rate of increase is constant during a woman's lifetime.¹⁵⁰

The existing data regarding HT in women experiencing menopause at the median age should not be extrapolated to women experiencing premature menopause and initiating HT at that time. The well-documented safety of supraphysiologic doses of HT in the form of oral contraceptives in young women suggests that physiologic dosing of HT for women with POI or premature menopause would convey minimal risk. Given the potential harmful effects of estrogen deficiency on bone mass in young women who may still be building their peak bone mass and the severity of vasomotor symptoms in younger women, the benefits of HT are potentially greater in this age group (see "Osteoporosis").

The lack of clinical trials on this topic necessitates clinical judgment. In the absence of contraindications, NAMS recommends the use of HT or oral contraceptives until the median age of natural menopause, with periodic reassessment.

Total mortality

The WHI trials are consistent with observational studies and meta-analyses¹⁵¹ indicating that HT may reduce total mortality when initiated soon after menopause. The WHI suggests that both ET and EPT nonsignificantly reduce total mortality by 30% when initiated in women younger than 60 years and that when data from the ET and EPT arms were combined, that reduction was statistically significant.³⁶ There were 10 fewer deaths per 10,000 women aged 50 to 59 years, compared with 16 additional deaths among those aged 70 to 79 years.³⁶ The mortality advantage for younger women did not remain significant when evaluated by years since menopause.³⁶

PRACTICAL THERAPEUTIC ISSUES

Class versus specific product effect

All estrogens have some common features and effects as well as potentially different properties. The same is true of all progestogens. However, in the absence of RCTs designed to compare clinical outcomes of various estrogens and progestogens, clinicians will be required to generalize the clinical trial results, tempered by emerging reports from observational studies (as addressed in individual sections of this report), for one agent to all agents within the same hormonal family. On a theoretical basis, however, there are likely to be differences within each family based on factors such as relative potency of the compound, androgenicity, glucocorticoid effects, bioavailability, and route of administration.

Progestogen indication

The primary menopause-related indication for progestogen use is to negate the increased risk of endometrial cancer from systemic ET use. All women with an intact uterus who use systemic ET should also be prescribed adequate progestogen. With occasional exceptions (eg, history of extensive endometriosis), postmenopausal women without a uterus should not be prescribed a progestogen with systemic ET.¹⁵²⁻¹⁵⁴

A progestogen is generally not indicated when ET is administered locally in a low dose for vaginal atrophy, although trials to date have been limited to only 1 year.¹⁵⁵ Although one 2-year study of the ultralow-dose estradiol patch found no statistically significant increase in endometrial hyperplasia,¹⁵⁶ intermittent progestogen probably should be used with long-term use of any systemic ET, including the ultralow-dose patch, which carries that recommendation in the product information sheet (see “Dose and route of administration”).

Concomitant progestogen may improve the efficacy of low-dose ET in treating vasomotor symptoms. Some women who use EPT may experience dysphoria from the progestogen component. A combination of estrogen with an estrogen agonist/antagonist is currently under investigation and may become an alternate option to progestogen.

Dose and route of administration

The lowest effective dose of estrogen consistent with treatment goals, benefits, and risks for the individual woman should be the therapeutic goal, with an appropriate dose of progestogen added to counter the adverse effects of systemic ET on the uterus. Among the lower doses typically used when initiating systemic ET are 0.3 mg to 0.45 mg oral CE, 0.5 mg oral micronized 17 β -estradiol, and 0.014 mg to 0.0375 mg transdermal 17 β -estradiol patch. Low-dose formulations of estradiol are available in approved topical gels, creams, and sprays. Estrogen doses less than those traditionally prescribed (<0.625 mg CE) often require longer duration of treatment upon initiation to achieve maximal efficacy in reducing vasomotor symptoms.^{157,158} Tailoring the dose to a woman's individual needs represents an appropriate strategy in HT management.

Lower HT doses generally have fewer adverse effects, such as breast tenderness and uterine bleeding, and may have a more favorable benefit-risk ratio than standard doses. In a nested case-control study from the UK General Practice Research database, the risk of stroke was not increased with low-dose transdermal estrogen (≤ 0.05 mg) but did increase with oral therapies and with higher transdermal doses.¹⁵⁹ Lower doses of HT have not been tested in long-term trials with clinical outcomes to support an assumed more favorable benefit-risk ratio.

All routes of administration of ET can effectively treat menopausal symptoms. Nonoral routes of administration including transdermal, vaginal, and intrauterine systems may offer both advantages and disadvantages compared with the oral route, but the long-term benefit-risk ratio has not been demonstrated in RCTs with clinical outcomes. There are differences related to the role of the first-pass hepatic effect, the hormone concentrations in the blood achieved by a given route, and the biologic activity of ingredients. With transdermal therapy, there is no significant increase in triglycerides, C-reactive protein, or sex hormone-binding globulin and little effect on blood pressure. With cutaneous therapies, caution should be exercised to avoid inadvertent transfer to children and animals.¹⁶⁰

There is growing observational evidence that transdermal ET may be associated with a lower risk of deep vein thrombosis, stroke, and MI.^{64,65,68,161}

There are multiple progestogen dosing-regimen options for endometrial safety. The dose varies based on the progestogen used and the estrogen dose, typically starting at the lowest effective doses of 1.5 mg medroxyprogesterone acetate, 0.1 mg norethindrone acetate, 0.5 mg drospirenone, or 100 mg micronized progesterone. Different doses may have different health outcomes. A long-term Finnish observational study reported that continuous use of EPT reduced the risk of endometrial neoplasia compared to no use of HT, and sequential progestogen therapy with ET increased the risk, particularly with long-cycle progestogen.¹⁶² In this study, all progestogens performed similarly within a given regimen.

Oral progestogens, combined with systemic estrogen, and combined progestogen-estrogen matrix patches have demonstrated endometrial protection and are government approved. A progestin-containing intrauterine system and a vaginal progesterone cream are government approved for use in premenopausal women; however, neither has been approved for use in postmenopausal women. A small study reported that when used with systemic ET in perimenopausal and postmenopausal women, the progestin-containing intrauterine system was found to provide endometrial protection equivalent to protection provided by systemic progestogen administered continuously and superior protection compared with progestogen given sequentially.¹⁶³

Bioidentical hormones

The term bioidentical hormones is most often used to describe custom-made HT formulations (called bioidentical hormone therapy [BHT]) that are compounded for an individual according to a healthcare provider's prescription. The term is used by proponents of BHT to convey that the hormones they use are identical to the hormones made by the ovaries. In that regard, the term can also be used to refer to many well-tested, government-approved, brand-name HT products containing hormones chemically identical to those produced by women (primarily in the ovaries), such as 17 β -estradiol and progesterone.

Custom-compounding of HT may combine several hormones (eg, estradiol, estrone, and estriol) and use nonstandard routes of administration (eg, subdermal implants). Some of the hormones are not government approved (estriol) or monitored and some of the compounded therapies contain non-hormonal ingredients (eg, dyes, preservatives) that some women cannot tolerate. Use of BHT has escalated in recent years, along with the use of salivary hormone testing, which has been proven to be inaccurate and unreliable. There may be increased risks to the women using these products. Custom-compounded formulations, including BHT, have not been tested for efficacy or safety; product information is not consistently provided to women along with their prescription, as is required with commercially available HT; and batch standardization and purity may be uncertain. The dosing of compounded progesterone is particularly difficult to assess because the levels in serum, saliva, and tissue are markedly different.¹⁶⁴ Custom-compounded drug formulations are not government approved.

The US Food and Drug Administration has ruled that some compounding pharmacies have made claims about the safety and effectiveness of BHT unsupported by clinical trial data and considered to be false and misleading.¹⁶⁵ Pharmacies have been instructed not to use estriol without an investigational new drug authorization. The Food and Drug Administration also states that there is no scientific basis for using saliva testing to adjust hormone levels.

NAMS recommends that BHT products include a patient package insert identical to that required for products that have government approval. In the absence of efficacy and safety data for BHT, the generalized benefit-risk ratio data of commercially available HT products should apply equally to BHT. For most women, government-approved HT will provide appropriate therapy without the risks of custom preparations. Therefore, NAMS does not generally recommend compounded EPT or ET unless necessary because of allergies to ingredients contained in government-approved products.

TREATMENT ISSUES

Duration of use

One of the most challenging issues regarding HT is the duration of use. Long-term follow-up data from the WHI have clarified the increased risk of breast cancer and breast cancer mortality with 4 to 5 years of EPT used at the time of menopause and a slightly later onset of breast cancer if used after a hiatus in estrogen exposure.^{74,78} Regarding ET, there was no increase in risk of breast cancer with early postmenopausal use in the WHI or NHS, and there was decrease in breast cancer incidence when used after a hiatus in estrogen exposure in the WHI.^{76,85} Long-term use of ET (15-20 y in the NHS) can be expected to increase breast cancer, but to a lesser degree than EPT.⁸⁵

Potential coronary artery disease and CHD benefits were also seen with early use of ET. In the WHI ET trial, women ages 50 to 59 years had a significantly lower risk of combined endpoints including CHD and total MI and no elevation in breast cancer risk.²⁸ Observational studies suggest that longer duration of HT use is associated with a reduced risk of CHD and related mortality.¹⁶⁶ The WHI RCTs and observational study suggest a pattern of lower risk of CHD among women who used HT for 5 or more years,⁴⁰ but this is not conclusive and should be considered in light of other factors altered by duration of therapy, such as breast cancer. In contrast, both ET and EPT are associated with an initial increase in CHD risk among women who are more distant from menopause at the time of HT initiation.^{38,167,168}

These findings allow for longer duration of use with ET based on a woman's symptoms, preferences, and current benefit-risk profile.

Provided that the woman is well aware of the potential benefits and risks and has clinical supervision, extending EPT use with the lowest effective dose is acceptable under some circumstances, including (1) for the woman who has determined that the benefits of menopause symptom relief outweigh risks, notably after failing an attempt to stop EPT, and (2) for the

woman at high risk of fracture for whom alternate therapies are not appropriate or cause unacceptable adverse effects.

Discontinuation of HT

Data from long-term follow-up of women who discontinued ET and EPT have increased our understanding of the sequelae of discontinuing HT. In the WHI, women in the EPT group who had stopped HT for 3 years had a rate of cardiovascular events, fractures, and colon cancers equivalent to that of women who had been assigned to placebo.²⁷ The only statistical difference was an increase in the rates of all cancer in women who had been assigned to EPT, with an excess of 30 cancers per 10,000 women per year of EPT, including a number of fatal lung cancers.^{27,77} For women without a uterus, when followed for 3 years after stopping ET, there was no overall increased or decreased risk of CHD, deep-vein thrombosis, stroke, hip fracture, colorectal cancer, or total mortality. A statistically significant decreased risk of invasive breast cancer persisted (8 fewer cases/10,000 women).²⁸ Discontinuance of HT will lead to a transient increased incidence of fracture, including hip fracture.¹⁶⁹ After 4 years of follow-up in the ET arm of the WHI, cumulative fracture rates were similar for both ET and placebo groups.²⁸

HRs for all-cause mortality, reflecting the balance of all of the above and other outcomes, tended to be neutral in both the EPT and ET arms of the WHI (HR, 0.98 and 1.04, respectively). During the 3-year postintervention phase of the EPT trial, mortality rates were borderline elevated (HR, 1.15; 95% CI, 0.95-1.39) primarily because of the aforementioned increase in cancer. During the entire EPT follow-up period (active treatment plus poststopping phases), the HR for all-cause mortality in the EPT arm was 1.04 (95% CI, 0.91-1.18)²⁷ and 1.02 (95% CI, 0.91-1.15) in the ET arm.²⁸

Regarding other outcomes after discontinuance of EPT, an initial analysis of data from the National Cancer Institute's Surveillance, Epidemiology, and End Results registries showed that the age-adjusted incidence rate of breast cancer in women in the United States fell sharply (by 6.7%) in 2003, as compared with the rate in 2002.¹⁷⁰ The decrease was evident only in women who were 50 years or older and was more evident in cancers that were estrogen receptor positive, which represent most breast cancers. It was theorized that the drop could be related to the large number of women discontinuing HT after the termination of the EPT arm of the WHI.

Vasomotor symptoms have an approximately 50% chance of recurring when HT is discontinued, independent of age and duration of use.^{171,172} In one RCT, tapering the dose of HT for 1 month and abruptly discontinuing HT had a similar impact on vasomotor symptoms.¹⁷³ The decision to continue HT should be individualized based on the severity of symptoms and current benefit-risk ratio considerations.

CONCLUSIONS AND RECOMMENDATIONS

- Individualization is of key importance in the decision to use HT and should incorporate the woman's health and

quality of life priorities as well as her personal risk factors, such as risk of venous thrombosis, CHD, stroke, and breast cancer.

- The recommendation for duration of therapy differs for EPT and ET. For EPT, duration is limited by the increased risk of breast cancer and breast cancer mortality associated with 3 to 5 years of use; for ET, a more favorable benefit-risk profile was observed during a mean of 7 years of use and 4 years of follow-up, a finding that allows more flexibility in duration of use.
- ET is the most effective treatment of symptoms of vulvar and vaginal atrophy; low-dose, local vaginal ET is advised when only vaginal symptoms are present.
- Women with premature or early menopause who are otherwise appropriate candidates for HT can use HT at least until the median age of natural menopause (age 51 y). Longer duration of treatment can be considered if needed for symptom management.
- Although ET did not increase breast cancer risk in the WHI, there is a lack of safety data supporting the use of ET in breast cancer survivors, and one RCT reported a higher increase in breast cancer recurrence rates.
- Both transdermal and low-dose oral estrogen have been associated with lower risks of VTE and stroke than standard doses of oral estrogen, but RCT evidence is not yet available.

SUMMARY

In the decade since the first publication of results from the WHI EPT study, much has been learned. There is a growing body of evidence that HT formulation, route of administration, and the timing of therapy produce different effects. Constructing an individual benefit-risk profile is essential for every woman considering any HT regimen. A woman's interest in using HT will vary depending on her individual situation, particularly the severity of her menopausal symptoms and their effect on her QOL. The absolute risks known to date for use of HT in healthy women ages 50 to 59 years are low. In contrast, long-term HT or HT initiation in older women is associated with greater risks.

Recommendations for duration of use differ between ET and EPT. Given the more favorable safety profile of ET, it could be considered for longer duration of therapy in the absence of adverse effects and risk factors. Women experiencing premature menopause are at increased risk of osteoporosis and, possibly, cardiovascular disease, and they often experience more intense symptoms than do women reaching menopause at the median age. Therefore, HT generally is advised for these young women until the median age of menopause when treatment should be reassessed.

Additional research is needed to understand the different effects of ET and EPT and how they apply to individual women. Further research is also needed to more clearly delineate the role of aging versus menopause and the effects of genetics,

lifestyle, and individual clinical characteristics on midlife women's health.

Acknowledgments: NAMS appreciates the contributions of the following members of the Advisory Panel: Co-Chairs: Margery L.S. Gass, MD, NCMP—Executive Director, The North American Menopause Society, Mayfield Heights, OH; Consultant, Cleveland Clinic Center for Specialized Women's Health, Cleveland, OH, and JoAnn E. Manson, MD, DrPH, NCMP—Professor of Medicine and the Michael and Lee Bell Professor of Women's Health, Harvard Medical School; Chief of Preventive Medicine, Co-Director, Connors Center for Women's Health and Gender Biology, Brigham and Women's Hospital, Boston, MA. Panelists: Felicia Cosman, MD—Professor of Clinical Medicine, Columbia University; Clinical Director, National Osteoporosis Foundation, Medical Director, Clinical Research Center, Helen Hayes Hospital, West Haverstraw, NY. Francine Grodstein, ScD—Associate Professor of Medicine, Channing Lab, Brigham and Women's Hospital, Harvard Medical School, Boston, MA. V. Craig Jordan, OBE, PhD, DSc, FmedSci—Scientific Director and Vice Chairman of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC. Richard H. Karas, MD, PhD—Professor of Medicine, Director, Women's Heart Center; Co-Director, Molecular Cardiology Research Center, Tufts University School of Medicine, Boston, MA. Andrew M. Kaunitz, MD—Professor and Associate Chair, Department of Obstetrics & Gynecology, Associate Program Director, Residency Program in Obstetrics & Gynecology, University of Florida College of Medicine, Jacksonville, FL. Pauline M. Maki, PhD—Associate Professor of Psychiatry and Psychology, Director, Women's Mental Health Research, University of Illinois at Chicago College of Medicine, Chicago, IL. Peter J. Schmidt, MD—Acting Chief, Behavioral Endocrinology Branch, National Institute of Mental Health, National Institutes of Health, Department of Health and Human Services, Bethesda, MD. Jan L. Shifren, MD, NCMP—Associate Professor of Obstetrics, Gynecology, and Reproductive Biology, Harvard Medical School; Director, Menopause Program, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, Boston, MA. Cynthia A. Stuenkel, MD, NCMP—Clinical Professor of Medicine, Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA. Wulf H. Utian, MD, PhD, DSc(Med), NCMP—Professor Emeritus, Case Western Reserve University School of Medicine, Executive Director Emeritus, The North American Menopause Society, Cleveland, OH.

The Society also acknowledges the editorial contributions of Kathryn J. Wisch, NAMS Managing Editor, and Angela M. Bilancini, NAMS Associate Medical Editor.

The position statement was reviewed and approved by the 2011-2012 NAMS Board of Trustees: Thomas B. Clarkson, DVM—Professor of Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, NC. Margery L.S. Gass, MD, NCMP—Executive Director, The North American Menopause Society, Mayfield Heights, OH; Consultant, Cleveland Clinic Center for Specialized Women's Health, Cleveland, OH. Steven R. Goldstein, MD, FACOG, CCD, NCMP—Professor of Obstetrics and Gynecology, New York University School of Medicine, New York, NY. Risa Kagan, MD, FACOG, CCD, NCMP—Clinical Professor, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, East Bay Physicians Medical Group, Berkeley, CA. Andrew M. Kaunitz, MD—Professor and Associate Chair, Department of Obstetrics and Gynecology, Associate Program Director, Residency Program in Obstetrics & Gynecology, University of Florida College of Medicine, Jacksonville, FL. Pauline M. Maki, PhD (Secretary)—Associate Professor of Psychiatry and Psychology, Director, Women's Mental Health Research, University of Illinois at Chicago College of Medicine, Chicago, IL. JoAnn E. Manson, MD, DrPH, NCMP (President)—Professor of Medicine and the Michael and Lee Bell

Professor of Women's Health, Harvard Medical School; Chief of Preventive Medicine, Co-Director, Connors Center for Women's Health and Gender Biology, Brigham and Women's Hospital, Boston, MA. Diane T. Pace, PhD, FNP, FAANP, NCMP (President-Elect)—Assistant Dean for Faculty Practice, Director, University Health Services, University of Tennessee Health Science Center, College of Nursing, Memphis, TN. Isaac Schiff, MD (Ex Officio)—Joe Vincent Meigs Professor of Gynecology, Harvard Medical School; Chief, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, Boston, MA. Peter F. Schnatz, DO, FACOG, FACP, NCMP—Associate Chairman and Residency Program Director, Department of Obstetrics and Gynecology, The Reading Hospital and Medical Center, Reading, PA. Marla Shapiro, MDCM, CCFP, MHSc, FRCPC(C), FCFP, NCMP—Associate Professor, Department of Family and Community Medicine, University of Toronto, Toronto, Ontario, Canada. Jan L. Shifren, MD, NCMP (Treasurer)—Associate Professor of Obstetrics, Gynecology, and Reproductive Biology, Harvard Medical School; Director, Menopause Program, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital, Boston, MA. Lynnette Leidy Sievert, PhD—Professor, Department of Anthropology, University of Massachusetts, Amherst, MA. Wulf H. Utian, MD, PhD, DSc(Med)—Professor Emeritus, Case Western Reserve University School of Medicine; Executive Director Emeritus, The North American Menopause Society, Cleveland, OH. Michelle P. Warren, MD, NCMP—Medical Director, Center for Menopause, Hormonal Disorders, and Women's Health; Professor of Medicine, Obstetrics and Gynecology, Wyeth Professor of Women's Health, Columbia Presbyterian Medical Center, Department of Obstetrics and Gynecology, New York, NY.

Financial disclosure/conflicts of interest: For the Advisory Panel, Dr. Cosman reports: Consultant/Advisory Board: Amgen, Eli Lilly, Merck, Novartis, Zosano; Grants/Research Support: Eli Lilly, Novartis; Speaker's Bureau: Amgen, Eli Lilly, Novartis; Dr. Gass reports: No significant financial relationships. Dr. Grodstein reports: No significant financial relationships. Dr. Jordan reports: No significant financial relationships. Dr. Karas reports: No significant financial relationships. Dr. Kaunitz reports: Consultant/Advisory Board: Bayer, Merck, Noven, Teva; Grants/Research Support: Bayer, Endoceutics, Medical Diagnostic Laboratories, Merck, Noven, Teva. Dr. Maki reports: Consultant/Advisory Board: Noven. Dr. Manson reports: No significant financial relationships. Dr. Schmidt reports: No significant financial relationships. Dr. Shifren reports: Consultant/Advisory Board: New England Research Institutes; Grants/Research Support: Boehringer Ingelheim. Dr. Stuenkel reports: Consultant/Advisory Board: Noven, Pharmavite. Dr. Utian reports: Consultant/Advisory Board: Bayer, Bionovo, Cleveland Clinic Foundation Innovations Center, Hygeia (Orcas Therapeutics), Lupin, Merck, Novogyne, Pharmavite.

For additional contributors, Ms. Bilancini and Ms. Wisch report no significant financial relationships.

For the NAMS Board of Trustees, Dr. Clarkson reports: Consultant/Advisory Board: Pfizer; Grants/Research Support: Merck, Pfizer. Dr. Gass reports: No significant financial relationships. Dr. Goldstein reports: Board of Directors/Trustees: NYU School of Medicine Alumni Corporation (President-Elect), American Institute of Ultrasound in Medicine, SonoSite (President); Consultant/Advisory Board: Amgen, Bayer, Cook Ob/Gyn, Philips, Ultrasound, Shionogi; Speaker's Bureau: Amgen, Warner Chilcott. Dr. Kagan reports: Consultant/Advisory Board: Amgen, Bionovo, Depomed, Foundation for Osteoporosis Research and Education/American Bone Health, Merck, Novo Nordisk, Own the Bone Advisory Board of the American Orthopedic Association, Pfizer, Shionogi; Grants/Research Support: Boehringer Ingelheim, Bionovo, Biosante, Depomed, Pfizer; Speaker's Bureau: Amgen, Novogyne, Novo Nordisk. Dr. Kaunitz reports: Consultant/Advisory Board: Bayer, Merck, Noven, Teva; Grants/Research Support: Bayer, Endoceutics, Medical Diagnostic Laboratories, Merck,

Noven, Teva. Dr. Maki reports: Consultant/Advisory Board: Noven. Dr. Manson reports: No significant financial relationships. Dr. Pace reports: Consultant/Advisory Board: Novo Nordisk; Speaker's Bureau: Novo Nordisk. Dr. Schiff reports: No significant financial relationships. Dr. Schnatz reports: Board of Directors/Trustees: FaithCare. Dr. Shapiro reports: Board of Directors/Trustees: Baycrest, Research Canada, Canadian Partnership Against Cancer, SIGMA Canadian Menopause Society; Employment: Consultant, CTV Canada AM, CTV National News, CTV NewsChannel, Parents Canada, Canadian Health & Lifestyle Columnist; Consultant/Advisory Board: Amgen, AstraZeneca, Novartis, Pfizer; Grants/Research Support: Amgen, Pfizer, SIGMA; Speaker's Bureau: Amgen, AstraZeneca, Bayer, GlaxoSmithKline, Merck, Novartis, Novo Nordisk, Pfizer, Sanofi Pasteur, Warner Chilcott; Website Writer: CTV.ca. Dr. Shifren reports: Consultant/Advisory Board: New England Research Institutes; Grants/Research Support: Boehringer Ingelheim. Dr. Sievert reports: No significant financial relationships. Dr. Utian reports: Consultant/Advisory Board: Bayer, Bionovo, Cleveland Clinic Foundation Innovations Center, Hygeia (Orcas Therapeutics), Lupin, Merck, Novogyne, Pharmavite. Dr. Warren reports: Consultant/Advisory Board: Pfizer, Yoplait; Grants/Research Support: Pfizer; Speaker's Bureau: Amgen, Ascend Therapeutics, Pfizer, Upsher Smith.

REFERENCES

1. Barnabei VM, Cochrane BB, Aragaki AK, et al, for the Women's Health Initiative Investigators. Menopausal symptoms and treatment-related effects of estrogen and progestin in the Women's Health Initiative. *Obstet Gynecol* 2005;105:1063-1073.
2. National Institutes of Health. State-of-the-Science Panel. National Institutes of Health State-of-the-Science Conference statement: management of menopause-related symptoms. *Ann Intern Med* 2005;142:1003-1013.
3. MacLennan A, Broadbent J, Lester S, Moore V. Oral oestrogen and combined oestrogen/progestogen therapy versus placebo for hot flushes. *Cochrane Database Sys Rev* 2004;4:CD002978.
4. Schiff I, Tulchinsky D, Cramer D, Ryan KJ. Oral medroxyprogesterone in the treatment of postmenopausal symptoms. *JAMA* 1980;244:1443-1445.
5. Suckling J, Kennedy R, Lethaby A, Roberts H. Local oestrogen for vaginal atrophy in postmenopausal women. *Cochrane Database Sys Rev* 2006;4:CD001500.
6. The North American Menopause Society. The role of local vaginal estrogen for treatment of vaginal atrophy in postmenopausal women: 2007 position statement of The North American Menopause Society. *Menopause* 2007;14:357-369.
7. Sturdee DW, Panay N, on behalf of the International Menopause Society Writing Group. Recommendations for the management of postmenopausal vaginal atrophy. *Climacteric* 2010;13:509-522.
8. Wierman ME, Nappi RE, Avis N, et al. Endocrine aspects of women's sexual function. *J Sex Med* 2010;7:561-585.
9. Gass M, Cochrane BB, Larson JC, et al. Patterns and predictors of sexual activity among women in the Hormone Therapy trials of the Women's Health Initiative. *Menopause* 2011;18:1160-1171.
10. Davis SR, Guay AT, Shifren JL, Mazer NA. Endocrine aspects of female sexual dysfunction. *J Sex Med* 2004;1:82-86.
11. Cody JD, Richardson K, Moehrer B, Hextall A, Glazener CMA. Oestrogen therapy for urinary incontinence in postmenopausal women. *Cochrane Database Sys Rev* 2009;4:CD001405.
12. Nelken RS, Ozel BZ, Leegant AR, Felix JC, Mishell DR. Randomized trial of estradiol vaginal ring versus oral oxybutynin for the treatment of overactive bladder. *Menopause* 2011;18:962-966.
13. Townsend MK, Curhan GC, Resnick NM, Grodstein F. The incidence of urinary incontinence across Asian, black, and white women in the United States. *Am J Obstet Gynecol* 2010;202:378.e1-378.e7.
14. Hendrix SL, Cochrane BB, Nygaard IE, et al. Effects of estrogen with and without progestin on urinary incontinence. *JAMA* 2005;293:935-948.
15. Grady D, Brown JS, Vittinghoff E, Applegate W, Varner E, Snyder T, for the HERS Research Group. Postmenopausal hormones and

- incontinence: the Heart and Estrogen/Progestin Replacement Study. *Obstet Gynecol* 2001;97:116-120.
16. Waetjen LE, Brown JS, Vittinghoff E, et al, for the Ultra Low Dose Transdermal estRogen Assessment (ULTRA) Study. The effect of ultralow-dose transdermal estradiol on urinary incontinence in postmenopausal women. *Obstet Gynecol* 2005;106:946-952.
17. Maalouf NM, Sato AH, Welch BJ, et al. Postmenopausal hormone use and the risk of nephrolithiasis: results from the Women's Health Initiative hormone therapy trials. *Arch Intern Med* 2010;170:1678-1685.
18. Raz R, Stamm WE. A controlled trial of intravaginal estrinol in postmenopausal women with recurrent urinary tract infections. *N Engl J Med* 1993;329:753-756.
19. Eriksen BC. A randomized, open, parallel-group study on the preventive effect of an estradiol-releasing vaginal ring (Estring) on recurrent urinary tract infections in postmenopausal women. *Am J Obstet Gynecol* 1999;80:1072-1079.
20. Barnabei VM, Grady D, Stovall DW, et al. Menopausal symptoms in older women and the effects of treatment with hormone therapy. *Obstet Gynecol* 2002;100:1209-1218.
21. Hlatky MA, Boothroyd D, Vittinghoff E, Sharp P, Whooley MA, for the Heart and Estrogen/progestin Replacement Study (HERS) Research Group. Quality-of-life and depressive symptoms in postmenopausal women after receiving hormone therapy: results from the Heart and Estrogen/Progestin Replacement Study (HERS) trial. *JAMA* 2002;287:591-597.
22. Hays J, Ockene JK, Brunner RL, et al, for the Women's Health Initiative Investigators. Effects of estrogen plus progestin on health-related quality of life. *N Engl J Med* 2003;348:1839-1854.
23. Brunner RL, Gass M, Aragaki A, et al, for the Women's Health Initiative Investigators. Effects of conjugated equine estrogen on health-related quality of life in postmenopausal women with hysterectomy: results from the Women's Health Initiative Randomized Clinical Trial. *Arch Intern Med* 2005;165:1976-1986.
24. Michael YL, Gold R, Manson JE, Keast EM, et al. Hormone therapy and physical function change among older women in the Women's Health Initiative: a randomized controlled trial. *Menopause* 2010;17:295-302.
25. Cauley JA, Robbins J, Chen Z, et al, for the Women's Health Investigators. Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative Randomized Trial. *JAMA* 2003;290:1729-1738.
26. Jackson RD, LaCroix AZ, Gass M, et al, for the Women's Health Investigators. Calcium plus vitamin D supplementation and the risk of fractures. *N Engl J Med* 2006;354:669-683.
27. Heiss G, Wallace R, Anderson GL, et al, for the WHI Investigators. Health risks and benefits 3 years after stopping randomized treatment with estrogen and progestin. *JAMA* 2008;299:1036-1045.
28. LaCroix AZ, Chlebowski RT, Manson JE, et al, for the WHI Investigators. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA* 2011;305:1305-1314.
29. Vesco KK, Marshall LM, Nelson HD, et al, for the Study of Osteoporotic Fractures. Surgical menopause and nonvertebral fracture risk among older U.S. women [e-pub]. *Menopause* 2011.
30. Jacoby VL, Grady D, Wactawski-Wende J, et al. Oophorectomy vs ovarian conservation with hysterectomy: cardiovascular disease, hip fracture, and cancer in the Women's Health Initiative Observational Study. *Arch Intern Med* 2011;171:760-768.
31. Ouyang P, Michos ED, Karas RH. Hormone replacement therapy and the cardiovascular system lessons learned and unanswered questions. *J Am Coll Cardiol* 2006;47:1741-1753.
32. Toh S, Hernández-Díaz S, Logan R, Rossouw JE, Hernán MA. Coronary heart disease in postmenopausal recipients of estrogen plus progestin therapy: does the increased risk ever disappear? A randomized trial. *Ann Intern Med* 2010;152:211-217.
33. Barrett-Connor E, Bush TL. Estrogen and coronary heart disease in women. *JAMA* 1991;265:1861-1867.
34. Matthews KA, Kuller LH, Wing RR, Meilahn EN, Plantinga P. Prior to use of estrogen replacement therapy, are users healthier than nonusers? *Am J Epidemiol* 1996;143:971-978.
35. Barrett-Connor E, Wenger NK, Grady D, et al. Coronary heart disease in women, randomized clinical trials, HERS and RUTH. *Maturitas* 1998;31:1-7.
36. Rossouw JE, Prentice RL, Manson JE, et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA* 2007;297:1465-1477.
37. Prentice RL, Langer R, Stefanick ML, et al, for Women's Health Initiative Investigators. Combined postmenopausal hormone therapy and cardiovascular disease: toward resolving the discrepancy between observational studies and the Women's Health Initiative clinical trial. *Am J Epidemiol* 2005;162:404-414.
38. Hsia J, Langer RD, Manson JE, et al, for the Women's Health Initiative Investigators. Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. *Arch Intern Med* 2006;166:357-365.
39. Salpeter SR, Walsh JM, Greyber E, Salpeter EE. Brief report: coronary heart disease events associated with hormone therapy in younger and older women. A meta-analysis. *J Gen Intern Med* 2006;21:363-366.
40. Prentice RL, Manson JE, Langer RD, et al. Benefits and risks of postmenopausal hormone therapy when it is initiated soon after menopause. *Am J Epidemiol* 2009;170:12-23.
41. Akhrass F, Evans AT, Wang Y, et al. Hormone replacement therapy is associated with less coronary atherosclerosis in postmenopausal women. *J Clin Endocrinol Metab* 2003;88:5611-5614.
42. Barrett-Connor E, Laughlin GA. Hormone therapy and coronary artery calcification in asymptomatic postmenopausal women: the Rancho Bernardo Study. *Menopause* 2005;12:40-48.
43. Schisterman EF, Gallagher AM, Bairey Merz CN, et al. The association of hormone replacement therapy and coronary calcium as determined by electron beam tomography. *J Womens Health Gend Based Med* 2002;11:631-638.
44. Manson JE, Allison MA, Rossouw JE, et al, for the WHI and WHI-CACS Investigators. Estrogen therapy and coronary-artery calcification. *N Engl J Med* 2007;356:2591-2602.
45. Tremollieres FA, Cigagna F, Alquier C, Cauneille C, Pouilles J, Ribot C. Effect of hormone replacement therapy on age-related increase in carotid artery intima-media thickness in postmenopausal women. *Atherosclerosis* 2000;153:81-88.
46. Grady D, Rubin SM, Petitti DB, et al. Hormone therapy to prevent disease and prolong life in postmenopausal women. *Ann Intern Med* 1992;117:1016-1037.
47. Barrett Connor E, Grady D. Hormone replacement therapy, heart disease, and other considerations. *Annu Rev Public Health* 1998;19:55-72.
48. Hodis HN, Mack WJ, Lobo RA, Shoupe D, et al, for the Estrogen in Prevention of Atherosclerosis Trial Research Group. Estrogen in the prevention of atherosclerosis: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 2001;135:939-953.
49. Angerer P, Stork S, Kothny W, Schmitt P, von Schacky C. Effect of oral postmenopausal hormone replacement on progression of atherosclerosis: a randomized, controlled trial. *Arterioscler Thromb Vasc Biol* 2001;21:262-268.
50. Wassertheil-Smoller S, Hendrix SL, Limacher M, et al, for the WHI Investigators. Effect of estrogen plus progestin on stroke in postmenopausal women. The Women's Health Initiative: a randomized trial. *JAMA* 2003;289:2673-2684.
51. Hendrix SL, Wassertheil-Smoller S, Johnson KC, et al, for the Women's Health Initiative Investigators. Effects of estrogen plus progestin on stroke in the Women's Health Initiative: a randomized trial. *Circulation* 2006;113:2425-2434.
52. Simon JA, Hsia J, Cauley JA, et al. Postmenopausal hormone therapy and risk of stroke: the Heart and Estrogen-progestin Replacement Study (HERS). *Circulation* 2001;103:638-642.
53. Viscoli CM, Brass LM, Kernan WN, Sarel PM, Suissa S, Horwitz RI. A clinical trial of estrogen-replacement therapy after ischemic stroke. *N Engl J Med* 2001;345:1243-1249.
54. Vickers MR, MacLennan AH, Lawton B, et al. Main morbidities recorded in Women's International Study of long Duration Oestrogen after Menopause (WISDOM): a randomised controlled trial of hormone replacement therapy in postmenopausal women. *BMJ* 2007;335:239-251.
55. Grodstein F, Manson JE, Stampfer MJ, Rexrode K. Postmenopausal hormone therapy and stroke: role of time since menopause and age at initiation of hormone therapy. *Arch Intern Med* 2008;168:861-866.
56. Arana A, Varas C, González-Pérez A, Gutiérrez L, Bjerrum L, García Rodríguez LA. Hormone therapy and cerebrovascular events: a population-based nested case-control study. *Menopause* 2006;13:730-736.
57. Lobo RA. The risk of stroke in postmenopausal women receiving hormonal therapy. *Climacteric* 2009;12:81-85.

58. Lisabeth L, Bushnell C. Stroke risk in women: the role of menopause and hormone therapy. *Lancet Neurol* 2012;11:82-91.
59. Canonico M, Oger E, Plu-Bureau G, et al, for the Estrogen and Thromboembolism Risk (ESTHER) Study Group. Hormone therapy and venous thromboembolism among postmenopausal women. *Circulation* 2007;115:840-845.
60. Cushman M, Kuller LH, Prentice R, et al, for the Women's Health Initiative Investigators. Estrogen plus progestin and risk of venous thrombosis. *JAMA* 2004;292:1573-1580.
61. Curb JD, Prentice RL, Bray PF, et al. Venous thrombosis and conjugated equine estrogen in women without a uterus. *Arch Intern Med* 2006;166:772-780.
62. Høibraaten E, Qvigstad E, Arnesen H, Larsen S, Wickstrøm E, Sandset PM. Increased risk of recurrent venous thromboembolism during hormone replacement therapy—results of the randomized, double-blind, placebo-controlled Estrogen in Venous Thromboembolism Trial (EVTET). *Thromb Haemost* 2000;84:961-967.
63. Herrington DM, Vittinghoff E, Howard TD, et al. Factor V Leiden, hormone replacement therapy, and risk of venous thromboembolic events in women with coronary disease. *Arterioscler Thromb Vasc Biol* 2002;22:1012-1017.
64. Scarabin PY, Oger E, Plu-Bureau G, on behalf of the ESTrogen and THromboEmbolic Risk (ESTHER) Study Group. Differential association of oral and transdermal oestrogen-replacement therapy with venous thromboembolism risk. *Lancet* 2003;362:428-432.
65. Canonico M, Plu-Bureau G, Lowe GD, Scarabin PY. Hormone replacement therapy and risk of venous thromboembolism in postmenopausal women: systematic review and meta-analysis. *BMJ* 2008;336:1227-1231.
66. Laliberte F, Dea K, Duh MS, Kahler KH, Rolli M, Lefebvre P. Does the route of administration for estrogen hormone therapy impact the risk of venous thromboembolism? Estradiol transdermal system versus oral estrogen-only hormone therapy. *Menopause* 2011;17:1052-1059.
67. Canonico M, Alhenc-Gelas M, Plu-Bureau G, Olie V, Scarabin PY. Activated protein C resistance among postmenopausal women using transdermal estrogens: importance of progestogen. *Menopause* 2010;17:1122-1127.
68. Canonico M, Plu-Bureau G, Scarabin PY. Progestogens and venous thromboembolism among postmenopausal women using hormone therapy. *Maturitas* 2011;70:354-360.
69. Margolis KL, Bonds DE, Rodabough RJ, et al. Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. *Diabetologia* 2004;47:1175-1187.
70. Kanaya AM, Herrington D, Vittinghoff E, et al. Glycemic effects of postmenopausal hormone therapy: the Heart and Estrogen/Progestin Replacement Study. A randomized, doubleblind, placebo-controlled trial. *Ann Intern Med* 2003;138:1-9.
71. Bonds DE, Lasser N, Qi L, et al. The effect of conjugated equine oestrogen on diabetes incidence: the Women's Health Initiative randomised trial. *Diabetologia* 2006;49:459-468.
72. Espeland MA, Hogan PE, Fineberg SE, et al. Effect of postmenopausal hormone therapy on glucose and insulin concentrations. *Diabetes Care* 1998;21:1589-1595.
73. Grady D, Gebretsadik T, Kerlikowske K, Ernster V, Petitti D. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. *Obstet Gynecol* 1995;85:304-313.
74. Chlebowski RT, Hendrix SL, Langer RD, et al, for the WHI Investigators. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. *JAMA* 2003;289:3243-3253.
75. Fournier A, Mesrine S, Boutron-Ruault MC, Clavel-Chapelon F. Estrogen-progestogen menopausal hormone therapy and breast cancer: does delay from menopause onset to treatment initiation influence risks? *J Clin Oncol* 2009;27:5138-5143.
76. Stefanick ML, Anderson GL, Margolis KL, et al, for the WHI Investigators. Effects of conjugated equine estrogens on breast cancer and mammography screening in postmenopausal women with hysterectomy. *JAMA* 2006;295:1647-1657.
77. Chlebowski RT, Schwartz AG, Wakelee H, et al, for the Women's Health Initiative Investigators. Oestrogen plus progestin and lung cancer in postmenopausal women (Women's Health Initiative trial): a post-hoc analysis of a randomised trial. *Lancet* 2009;374:1243-1251.
78. Chlebowski RT, Anderson GL, Gass M, et al. Estrogen plus progestin and breast cancer incidence and mortality in postmenopausal women. *JAMA* 2010;304:1684-1692.
79. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA* 2002;288:321-333.
80. Prentice RL, Chlebowski RT, Stefanick ML, et al. Conjugated equine estrogens and breast cancer risk in the Women's Health Initiative Clinical Trial and Observational Study. *Am J Epidemiol* 2008;167:1407-1415.
81. Anderson GL, Chlebowski RT, Rossouw JE, et al. Prior hormone therapy and breast cancer risk in the Women's Health Initiative randomized trial of estrogen plus progestin. *Maturitas* 2006;55:103-115.
82. Beral V, Reeves G, Bull D, Green J, for the Million Women Study Collaborators. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. *J Natl Cancer Inst* 2011;103:296-305.
83. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a 'new' biology of estrogen-induced apoptosis. *Cancer Prev Res (Phila)* 2011;4:633-637.
84. Colditz GA, Hankinson SE, Hunter DJ, et al. The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. *N Engl J Med* 1995;332:1589-1593.
85. Colditz GA, Rosner B. Cumulative risk of breast cancer to age 70 years according to risk factor status: data from the Nurses' Health Study. *Am J Epidemiol* 2000;152:950-964.
86. Chen W, Manson J, Hankinson S, et al. Unopposed estrogen therapy and the risk of invasive breast cancer. *Arch Intern Med* 2006;166:1027-1032.
87. World Health Organization Collaborative Study of Cardiovascular Disease and Steroid Hormone Contraception. Acute myocardial infarction and combined oral contraceptives: results of an international multicentre case-control study. *Lancet* 1997;349:1202-1209.
88. Col NF, Kim JA, Chlebowski RT. Menopausal hormone therapy after breast cancer: a meta-analysis and critical appraisal of the evidence. *Breast Cancer Res* 2005;7:R535-R540.
89. Duma E, Wren B, Heller G, Leader L, Sjoblom P, Eden J. Hormone replacement therapy after a diagnosis of breast cancer: cancer recurrence and mortality. *Med J Aust* 2002;177:347-351.
90. Decker DA, Pettinga JE, VanderVelde N, Huang RR, Kestin L, Burdakin JH. Estrogen replacement therapy in breast cancer survivors: a matched-controlled series. *Menopause* 2003;10:277-285.
91. Beckmann MW, Jap D, Djahansouzi S, et al. Hormone replacement therapy after treatment of breast cancer: effects on postmenopausal symptoms, bone mineral density and recurrence rates. *Oncology* 2001;60:199-206.
92. Marttunen M, Hietanen P, Pyrhonen S, Tiitinen A, Ylikorkala O. A prospective study on women with a history of breast cancer and with or without estrogen replacement therapy. *Maturitas* 2001;39:217-225.
93. DiSaia P, Brewster W, Ziogas A, Anton-Culver H. Breast cancer survival and hormone replacement therapy: a cohort analysis. *Am J Clin Oncol* 2000;23:541-545.
94. Ursic-Vrscaj M, Bebar S. A case-control study of hormone replacement therapy after primary surgical breast cancer treatment. *Eur J Surg Oncol* 1999;25:146-151.
95. Holmberg L, Iversen OE, Rudenstam CM, et al, for the HABITS Study Group. Increased risk of recurrence after hormone replacement therapy in breast cancer survivors. *J Natl Cancer Inst* 2008;100:475-482.
96. Kaufman DW, Kelly JP, Welch WR, et al. Noncontraceptive estrogen use and epithelial ovarian cancer. *Am J Epidemiol* 1989;130:1142-1151.
97. Sit AS, Modugno F, Weissfeld JL, Berga SL, Ness RB. Hormone replacement therapy formulations and risk of epithelial ovarian carcinoma. *Gynecol Oncol* 2002;86:118-123.
98. Weiss NS, Lyon JL, Krishnamurthy S, Dietert SE, Liff JM, Daling JR. Noncontraceptive estrogen use and the occurrence of ovarian cancer. *J Natl Cancer Inst* 1982;68:95-98.
99. Purdie DM, Bain CJ, Siskind V, et al. Hormone replacement therapy and risk of epithelial ovarian cancer. *Br J Cancer* 1999;81:559-563.
100. Risch HA. Estrogen replacement therapy and risk of epithelial ovarian cancer. *Gynecol Oncol* 1996;63:254-257.
101. Rodriguez C, Patel AV, Calle EE, Jacob EJ, Thun MJ. Estrogen replacement therapy and ovarian cancer mortality in a large prospective study of US women. *JAMA* 2001;285:1460-1465.

102. Lacey JV Jr, Mink PJ, Lubin JH, et al. Menopausal hormone replacement therapy and risk of ovarian cancer. *JAMA* 2002;288:334-341.
103. Riman T, Dickman PW, Nilsson S, et al. Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in Swedish women. *J Natl Cancer Inst* 2002;94:497-504.
104. Glud E, Kjaer SK, Thomsen BL, et al. Hormone therapy and the impact of estrogen intake on the risk of ovarian cancer. *Arch Intern Med* 2004;164:2253-2259.
105. Moorman PG, Schildkraut JM, Calingaert B, Halabi S, Berchuck A. Menopausal hormones and risk of ovarian cancer. *Am J Obstet Gynecol* 2005;76-82.
106. Folsom AR, Anderson JP, Ross JA. Estrogen replacement therapy and ovarian cancer. *Epidemiology* 2004;15:100-104.
107. Lacey JV Jr, Brinton LA, Leitzmann, et al. Menopausal hormone therapy and ovarian cancer risk in the National Institutes of Health-AARP Diet and Health Study Cohort. *J Natl Cancer Inst* 2006;98:1397-1405.
108. Rossing MA, Cushing-Haugen KL, Wicklund KG, Doherty JA, Weiss NS. Menopausal hormone therapy and risk of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2007;16:2548-2556.
109. Mørch LS, Løkkegaard E, Andreasen AH, Krüger-Kjaer S, Lidegaard O. Hormone therapy and ovarian cancer. *JAMA* 2009;302:298-305.
110. Greiser CM, Greiser EM, Dören M. Menopausal hormone therapy and risk of ovarian cancer: systematic review and meta-analysis. *Hum Reprod Update* 2007;13:453-463.
111. Zhou B, Sun Q, Cong R, et al. Hormone replacement therapy and ovarian cancer risk: a meta-analysis. *Gynecol Oncol* 2008;108:641-651.
112. Anderson GL, Judd HL, Kaunitz AM, et al, for the Women's Health Initiative Investigators. Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures. The Women's Health Initiative Randomized Trial. *JAMA* 2003;290:1739-1748.
113. Chlebowski RT, Anderson GL, Manson JE, et al. Lung cancer among postmenopausal women treated with estrogen alone in the Women's Health Initiative Randomized Trial. *J Natl Cancer Inst* 2010;102:1-9.
114. Rodriguez C, Spencer Feigelson H, et al. Postmenopausal hormone therapy and lung cancer risk in the cancer prevention study II nutrition cohort. *Cancer Epidemiol Biomarkers Prev* 2008;17:655-660.
115. Ramnath N, Menezes RJ, Loewen G, et al. Hormone replacement therapy as a risk factor for non-small cell lung cancer: results of a case-control study. *Oncology* 2007;73:305-310.
116. Schwartz AG, Wenzlaff AS, Prysak GM, et al. Reproductive factors, hormone use, estrogen receptor expression and risk of non small-cell lung cancer in women. *J Clin Oncol* 2007;25:5785-5792.
117. Elliott AM, Hannaford PC. Use of exogenous hormones by women and lung cancer: evidence from the Royal College of General Practitioners' Oral Contraception Study. *Contraception* 2006;73:331-335.
118. Liu Y, Inoue M, Sobue T, Tsugane S. Reproductive factors, hormone use and the risk of lung cancer among middle-aged never-smoking Japanese women: a large-scale population-based cohort study. *Int J Cancer* 2005;117:662-666.
119. Schabath MB, Wu X, Vassilopoulou-Sellin R, Vaporciyan AA, Spitz MR. Hormone replacement therapy and lung cancer risk: a case-control analysis. *Clin Cancer Res* 2004;10:113-123.
120. Kreuzer M, Gerken M, Heinrich J, Kreienbrock L, Wichmann HE. Hormonal factors and risk of lung cancer among women? *Int J Epidemiol* 2003;32:263-271.
121. Olsson H, Bladström A, Ingvar C. Are smoking-associated cancers prevented or postponed in women using hormone replacement therapy? *Obstet Gynecol* 2003;102:565-570.
122. Taioli E, Wynder EL. Endocrine factors and adenocarcinoma of the lung in women. *J Natl Cancer Inst* 1994;86:869-870.
123. Slatore CG, Chien JW, Au DH, Satia JA, White E. Lung cancer and hormone replacement therapy: association in the vitamins and lifestyle study. *J Clin Oncol* 2010;28:1540-1546.
124. Greiser CM, Greiser EM, Dören M. Menopausal hormone therapy and risk of lung cancer—systematic review and meta-analysis. *Maturitas* 2010;65:198-204.
125. Oh SW, Myung SK, Park JY, Lym YL, Ju W. Hormone therapy and risk of lung cancer: a meta-analysis. *J Womens Health (Larchmt)* 2010;19:279-288.
126. Joffe H, Petrillo LF, Koukopoulos A, et al. Increased estradiol and improved sleep, but not hot flashes, predict enhanced mood during the menopausal transition. *J Clin Endocrinol Metab* 2011;96:E1044-E1054.
127. Schmidt PJ, Nieman L, Danaceau MA, et al. Estrogen replacement in perimenopause-related depression: a preliminary report. *Am J Obstet Gynecol* 2000;183:414-420.
128. Soares CN, Almeida OP, Joffe H, Cohen LS. Efficacy of estradiol for the treatment of depressive disorders in perimenopausal women: a double-blind, randomized, placebo-controlled trial. *Arch Gen Psychiatry* 2001;58:529-534.
129. Morrison MF, Kallan MJ, Ten Have T, Katz I, Tweedy K, Battistini M. Lack of efficacy of estradiol for depression in postmenopausal women: a randomized, controlled trial. *Biol Psychiatry* 2004;55:406-412.
130. Rasgon NL, Dunkin J, Fairbanks L, et al. Estrogen and response to sertraline in postmenopausal women with major depressive disorder: a pilot study. *J Psychiatr Res* 2007;41:338-343.
131. Cohen LS, Soares CN, Vitonis AF, Otto MW, Harlow BL. Risk for new onset of depression during the menopausal transition: the Harvard study of moods and cycles. *Arch Gen Psychiatry* 2006;63:385-390.
132. Phillips SM, Sherwin BB. Effects of estrogen on memory function in surgically menopausal women. *Psychoneuroendocrinology* 1992;17:485-495.
133. Sherwin BB. Estrogen and/or androgen replacement therapy and cognitive functioning in surgically menopausal women. *Psychoneuroendocrinology* 1988;13:345-357.
134. Henderson VW. Gonadal hormones and cognitive aging: a midlife perspective. *Womens Health (Lond Engl)* 2011;7:81-93.
135. Greendale GA, Huang MH, Wight RG, et al. Effects of the menopause transition and hormone use on cognitive performance in midlife women. *Neurology* 2009;72:1850-1857.
136. Greendale GA, Wight RG, Huang MH, et al. Menopause-associated symptoms and cognitive performance: results from the study of women's health across the nation. *Am J Epidemiol* 2010;171:1214-1224.
137. Kang JH, Weuve J, Grodstein F. Postmenopausal hormone therapy and risk of cognitive decline in community-dwelling aging women. *Neurology* 2004;63:101-107.
138. Binder EF, Schechtman KB, Birge SJ, Williams DB, Kohrt WM. Effects of hormone replacement therapy on cognitive performance in elderly women. *Maturitas* 2001;38:137-146.
139. Grady D, Yaffe K, Kristof M, Lin F, Richards C, Barrett-Connor E. Effect of postmenopausal hormone therapy on cognitive function: the Heart and Estrogen/progestin Replacement Study. *Am J Med* 2002;113:543-548.
140. Resnick SM, Maki PM, Rapp SR, et al. Effects of combination estrogen plus progestin hormone treatment on cognition and affect. *J Clin Endocrinol Metab* 2006;91:1802-1810.
141. Shumaker S, Legault C, Rapp S, et al. Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: The Women's Health Initiative Memory Study: a randomized controlled trial. *JAMA* 2003;289:2651-2662.
142. Resnick SM, Espeland MA, An Y, et al. Effects of conjugated equine estrogens on cognition and affect in postmenopausal women with prior hysterectomy. *J Clin Endocrinol Metab* 2009;94:4152-4161.
143. Yaffe K, Sawaya G, Lieberburg I, Grady D. Estrogen therapy in postmenopausal women: Effects on cognitive function and dementia. *JAMA* 1998;279:688-695.
144. Rocca WA, Bower JH, Maraganore DM, et al. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. *Neurology* 2007;69:1074-1083.
145. Henderson VW, Sherwin BB. Surgical versus natural menopause: cognitive issues. *Menopause* 2007;14:572-579.
146. Luoma P, Melberg A, Rinne JO, et al. Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. *Lancet* 2004;364:875-882.
147. Parker WH, Broder MS, Chang E, et al. Ovarian conservation at the time of hysterectomy and long-term health outcomes in the Nurses' Health Study. *Obstet Gynecol* 2009;113:1027-1037.
148. Kok HS, van Asselt KM, van der Schouw YT, et al. Heart disease risk determines menopausal age rather than the reverse. *J Am Coll Cardiol* 2006;47:1976-1983.
149. Gold EB, Bromberger J, Crawford S, et al. Factors associated with age at natural menopause in a multiethnic sample of midlife women. *Am J Epidemiol* 2001;153:865-874.
150. Vaidya D, Becker DM, Bittner V, Mathias RA, Ouyang P. Ageing, menopause, and ischaemic heart disease mortality in England, Wales, and the United States: modelling study of national mortality data. *BMJ* 2011;343:d5170.

151. Salpeter SR, Cheng J, Thabane L, Buckley NS, Salpeter EE. Bayesian meta-analysis of hormone therapy and mortality in younger postmenopausal women. *Am J Med* 2009;122:1016-1022.
152. Efthymiou CA. Endometriosis-associated intestinal tumours: a consequence of long-term unopposed oestrogen? *Ann R Coll Surg Engl* 2009;91:259-260.
153. Giudice LC. Clinical practice. Endometriosis. *N Engl J Med* 2010;362:2389-2398.
154. Pachman DR, Jones JM, Loprinzi CL. Management of menopause-associated vasomotor symptoms: Current treatment options, challenges and future directions. *Int J Womens Health* 2010;2:123-135.
155. Ettinger B, Ensrud KE, Wallace R, et al. Effects of ultralow-dose transdermal estradiol on bone mineral density: a randomized clinical trial. *Obstet Gynecol* 2004;104:443-451.
156. Johnson R, Ettinger B, Macer J, et al. Uterine and vaginal effects of unopposed ultralow-dose transdermal estradiol. *Obstet Gynecol* 2005;105:779-787.
157. Utian WH, Shoupe D, Bachmann G, Pinkerton JV, Pickar JH. Relief of vasomotor symptoms and vaginal atrophy with lower doses of conjugated equine estrogens and medroxyprogesterone acetate. *Fertil Steril* 2001;75:1065-1079.
158. Bachmann GA, Schaefer M, Uddin A, Utian WH. Lowest effective transdermal 17 β -estradiol dose for relief of hot flashes in postmenopausal women: a randomized controlled trial. *Obstet Gynecol* 2007;110:771-779.
159. Renoux C, Dell'aniello S, Garbe E, Suissa S. Transdermal and oral hormone replacement therapy and the risk of stroke: a nested case-control study. *BMJ* 2010;340:c2519.
160. Steunkel CA, Gass M. Results from 2010 NAMS Survey on secondary transfer of transdermal estrogen preparations: Abstract P-85. *Menopause* 2011;18:1371.
161. Lokkegaard E, Andreasen AH, Jacobsen RK, Nielsen LH, Agger C, Lidegaard O. Hormone therapy and risk of myocardial infarction: a national register study. *Eur Heart J* 2008;29:2660-2668.
162. Jaakkola S, Lyytinen H, Pukkala E, Ylikorkala O. Endometrial cancer in postmenopausal women using estradiol-progestin therapy. *Obstet Gynecol* 2009;114:1197-1204.
163. Somboonporn W, Panna S, Temtanakitpaisan T, Kaewrudee S, Soontrapa S. Effects of the levonorgestrel-releasing intrauterine system plus estrogen therapy in perimenopausal and postmenopausal women: systematic review and meta-analysis. *Menopause* 2011;18:1060-1066.
164. Bhavnani BR, Stanczyk FZ. Misconception and concerns about bio-identical hormones used for custom-compounded hormone therapy [e-pub]. *J Clin Endocrinol Metab* 2011.
165. Food and Drug Administration. FDA takes action against compounded menopause hormone therapy drugs. 2008. Available at: <http://www.fda.gov/newsevents/newsroom/pressannouncements/2008/ucm116832.htm>. Accessed January 10, 2012.
166. Grodstein F, Manson JE, Colditz GA, et al. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med* 2000;133:933-941.
167. Hulley S, Grady D, Bush T, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 1998;280:605-613.
168. Manson JE, Hsia J, Johnson KC, et al, for the Women's Health Initiative Investigators. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 2003;349:523-534.
169. Karim R, Dell RM, Greene DF, Mack WJ, Gallagher JC, Hodis HN. Hip fracture in postmenopausal women after cessation of hormone therapy: results from a prospective study in a large health management organization. *Menopause* 2011;18:1172-1177.
170. National Cancer Institute. Surveillance, Epidemiology, and End Results (SEER). Available at: <http://seer.cancer.gov/statfacts/html/breast.html>. Accessed January 10, 2012.
171. Ockene JK, Barad DH, Cochrane BB, et al. Symptom experience after discontinuing use of estrogen plus progestin. *JAMA* 2005;294:183-193.
172. Brunner RL, Aragaki A, Barnabei V, et al. Menopausal symptom experience before and after stopping estrogen therapy in the Women's Health Initiative randomized, placebo-controlled trial. *Menopause* 2010;17:946-954.
173. Haimov-Kochman R, Barak-Glantz E, et al. Gradual discontinuation of hormone therapy does not prevent the reappearance of climacteric symptoms: a randomized prospective study. *Menopause* 2006;13:370-376.



Differential DNase I hypersensitivity reveals factor-dependent chromatin dynamics

Housheng Hansen He, Clifford A. Meyer, Mei Wei Chen, et al.

Genome Res. published online April 16, 2012

Access the most recent version at doi:[10.1101/gr.133280.111](https://doi.org/10.1101/gr.133280.111)

Supplemental Material <http://genome.cshlp.org/content/suppl/2012/03/19/gr.133280.111.DC1.html>

P<P Published online April 16, 2012 in advance of the print journal.

Creative Commons License This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see <http://genome.cshlp.org/site/misc/terms.xhtml>). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported License), as described at <http://creativecommons.org/licenses/by-nc/3.0/>.

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#)

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

To subscribe to *Genome Research* go to:
<http://genome.cshlp.org/subscriptions>

Research

Differential DNase I hypersensitivity reveals factor-dependent chromatin dynamics

Housheng Hansen He,^{1,2,5} Clifford A. Meyer,^{1,5} Mei Wei Chen,^{2,3} V. Craig Jordan,⁴ Myles Brown,^{2,3,6} and X. Shirley Liu^{1,3,6}

¹Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute and Harvard School of Public Health, Boston, Massachusetts 02115, USA; ²Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115, USA; ³Center for Functional Cancer Epigenetics, Dana-Farber Cancer Institute, Boston, Massachusetts 02215, USA; ⁴Department of Oncology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20057, USA

Transcription factor cistromes are highly cell-type specific. Chromatin accessibility, histone modifications, and nucleosome occupancy have all been found to play a role in defining these binding locations. Here, we show that hormone-induced DNase I hypersensitivity changes (Δ DHS) are highly predictive of androgen receptor (AR) and estrogen receptor 1 (ESR1) binding in prostate cancer and breast cancer cells, respectively. While chromatin structure prior to receptor binding and nucleosome occupancy after binding are strikingly different for ESR1 and AR, Δ DHS is highly predictive for both. AR binding is associated with changes in both local nucleosome occupancy and DNase I hypersensitivity. In contrast, while global ESR1 binding is unrelated to changes in nucleosome occupancy, DNase I hypersensitivity dynamics are also predictive of the ESR1 cistrome. These findings suggest that AR and ESR1 have distinct modes of interaction with chromatin and that DNase I hypersensitivity dynamics provides a general approach for predicting cell-type specific cistromes.

[Supplemental material is available for this article.]

In eukaryotes, transcription is regulated in a cell-type and condition-specific manner through the association of transcription factors with chromatin. The genome-wide binding sites of transcription factors, or the transcription factor cistromes, are influenced by the active protein levels of the transcription factors, chromatin structure, and DNA sequence. The nucleosome is the fundamental unit of chromatin structure and has been thought to compete with transcription factors for occupancy at thermodynamically favorable genomic loci. By comparing nucleosome occupancy maps generated from nucleosome-resolution H3K4me2 ChIP-seq, we found that nucleosome occupancy changes can be predictive of transcription factor cistromes. In particular, the binding of androgen receptor (AR) in prostate cancer LNCaP cells leads to an increased occupancy of nucleosomes flanking the AR binding site and decreased nucleosome occupancy in the position of the binding site itself (He et al. 2010). This approach also correctly predicted the binding of two factors, POU2F1 and NKX3-1, which are part of the secondary cellular response to androgens (He et al. 2010). This phenomenon is not unique to the LNCaP AR system; it has also been observed with CDX2, *HNF4A*, and GATA6 binding in intestinal differentiation (Verzi et al. 2010) and with GATA1 in hematopoiesis (Hu et al. 2011).

DNase I hypersensitivity is an alternative measure of chromatin accessibility (Wu 1980). DNase I hypersensitive sites (DHS), short regions of chromatin that are highly sensitive to cleavage by DNase I, typically occur in nucleosome free regions and frequently arise as a result of transcription factor binding. DNase I digestion followed by high-throughput sequencing (DNase-seq) has evolved into a powerful technique for identifying genome-wide DNase

hypersensitive sites (Ling et al. 2010; John et al. 2011; Siersbaek et al. 2011). Because transcription factor binding sites tend to be DNase I hypersensitive and DNase-seq does not require a factor-specific antibody, DNA sequence motif analysis on DHS data has been proposed as a method for discovering the binding sites of multiple transcription factors in a single experiment (Pique-Regi et al. 2011; Song et al. 2011).

To analyze the effects of androgen receptor (AR) and estrogen receptor 1 (ESR1) binding on DHS, we conducted genome-wide DNase-seq in both unstimulated and hormone-stimulated conditions. Using a quantitative measurement of DHS changes (Δ DHS) between these conditions, we were able to predict the ESR1 and AR cistromes. Although they are related members of the steroid receptor family, AR and ESR1 display distinct DHS profiles. Binding of both ESR1 and AR are frequently associated with significant increases in DHS signal upon hormone stimulation; however, ESR1 sites show strong DHS prior to binding and AR sites do not. Following hormone stimulation, FOXA1 binding sites that lacked AR or ESR1 binding are associated with a significant decrease in DHS. In MCF-7 cells, this change in DHS is linked not to a change in FOXA1 binding but rather to a decrease in the binding of the ESR1 coactivator, NCOA3, supporting a model of physiologic squelching. This study demonstrates that Δ DHS is a more effective and general approach to predict perturbation-induced transcription factor binding sites than either static DHS or nucleosome resolution H3K4me2 ChIP-seq.

Results

Estrogen receptor binding in breast cancer cells is not associated with significant nucleosome depletion

Based on our earlier work demonstrating the association between AR binding and nucleosome depletion (He et al. 2010), we carried out an H3K4me2 ChIP-seq experiment on MNase digested chromatin in the MCF-7 breast cancer cell line comparing unstimulated

⁵These authors contributed equally to this work.

⁶Corresponding authors.

E-mail myles_brown@dfci.harvard.edu.

E-mail xsliu@jimmy.harvard.edu.

Article published online before print. Article, supplemental material, and publication date are at <http://www.genome.org/cgi/doi/10.1101/gr.133280.111>.

(Veh) cells with cells grown under conditions of estrogen stimulation (E2). Consistent with previous studies (Barski et al. 2007; He et al. 2010), the H3K4me2 sites in both samples were mainly located in intergenic and intronic regions (Fig. 1A). Over 64% of estrogen receptor 1 (ESR1) binding sites overlapped with regions enriched in H3K4me2 (estrogen-stimulated) (Fig. 1B). We examined the distribution of H3K4me2 signals relative to the center of all ESR1 binding sites. Although in some cases ESR1 binds to regions depleted of H3K4me2 signal (Supplemental Fig. 1A), in both the vehicle and stimulated conditions the overall pattern shows a peak in the H3K4me2 signal that overlaps with the ESR1 binding sites (Fig. 1C).

We systematically assessed ESR1 binding as a function of the nucleosome stabilization-destabilization (NSD) score, a measure of nucleosome occupancy changes established in previous studies (He et al. 2010). The fraction of ESR1 binding sites located in high NSD scoring regions was no greater than the fraction in regions with low NSD scores (Fig. 1D). This pattern is significantly different from that observed in AR binding (Supplemental Fig. 1B). In AR binding an H3K4me2 tag density peak at the AR binding site becomes a trough after androgen stimulation, resulting in high NSD scoring regions being highly predictive of AR binding (He et al. 2010). Whereas in MCF-7 the distributions of NSD scores at ER and

non-ER sites are not significantly different (Supplemental Fig. 1C, P -value = 0.25), the distributions of NSD scores in LNCaP AR and non-AR sites are significantly different (Supplemental Fig. 1D, P -value = 2.2×10^{-16}).

In order to determine whether the differences in the behavior of AR in LNCaP cells and ESR1 in MCF7 cells were due to a difference in the transcription factors or the cell lines, we analyzed H3K4me2 enrichment at AR, ESR1, and FOXA1 sites together (Fig. 2A,B; Supplemental Fig. 2A,B). We included the winged helix transcription factor FOXA1 in the analysis as it acts as a “pioneer factor” in breast cancer cells and is required for ESR1 binding to a large proportion of its binding sites (Carroll et al. 2005; Lupien et al. 2008). The role of FOXA1 in AR action in prostate cancer cells is more complex, though a significant number of AR-bound sites are also bound by FOXA1 (Lupien et al. 2008; Wang et al. 2011). Consistent with our previous findings (He et al. 2010), sites bound by FOXA1 alone in either LNCaP or MCF7 cells show a pair of stimulus-independent peaks that flank a trough directly over the FOXA1 binding site (Fig. 2A,B, right panels).

When we examined the H3K4me2 signal at sites bound by AR or ESR1 that lacked FOXA1, we observed very different patterns. In LNCaP cells, AR binding sites that do not bind FOXA1 had a broad peak of H3K4me2 prior to hormone stimulation that resolved into

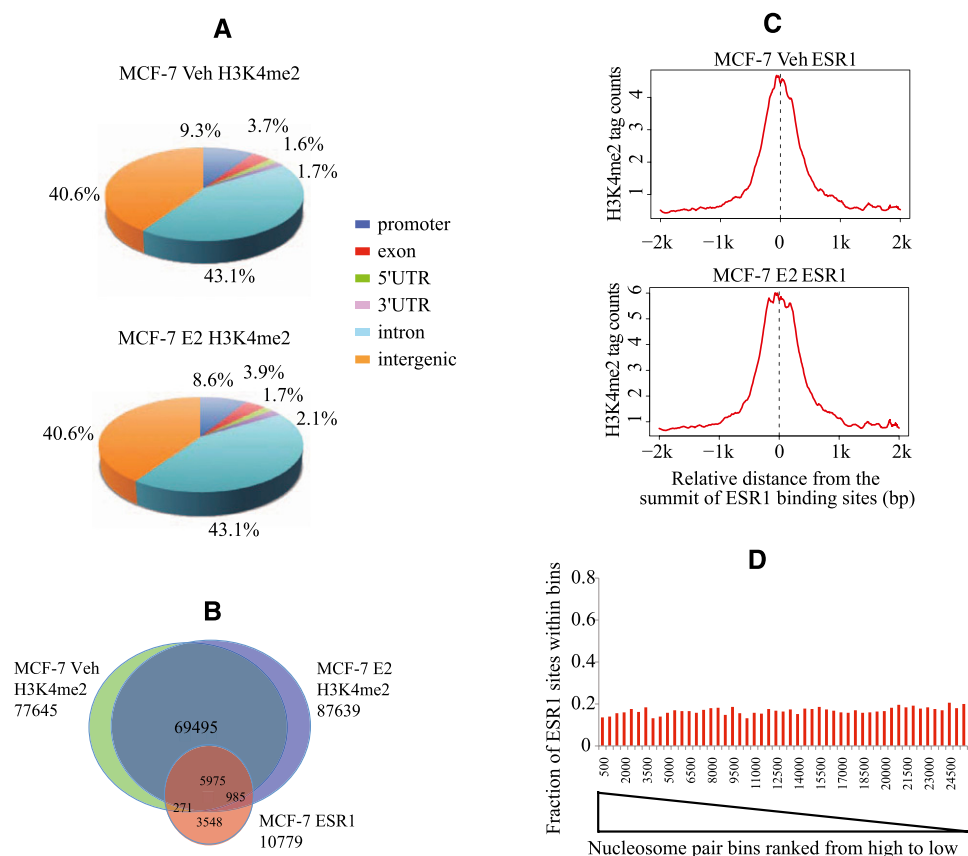


Figure 1. Characteristics of H3K4me2 ChIP-seq in MCF-7 cells. (A) Location of H3K4me2 ChIP-enriched peaks relative to gene annotations in unstimulated (Veh) and estrogen-stimulated (E2) conditions. (B) Venn diagram of ESR1 binding loci in relation to H3K4me2-enriched regions. (C) Distribution of H3K4me2 ChIP-seq signal at non-promoter (>1 kb from TSSs) ESR1 binding sites under unstimulated and estrogen-stimulated conditions. (D) The fraction of ESR1 binding sites in paired nucleosome bins sorted in descending order by NSD score (stimulated vs. unstimulated). Paired nucleosome regions are ranked by the NSD score that represents the differences in the H3K4me2 tag counts before and after estrogen treatment. These ranked regions are grouped into bins of 500 to calculate the proportion of real binding sites as a function of rank. (Y-axis) Fraction of the regions in each bin that overlap with ESR1 ChIP-seq enriched regions.

two sharp peaks flanking the AR binding site upon AR activation (Fig. 2A, left panels). In contrast, ESR1 binding sites in MCF7 cells that lack FOXA1 had a broad peak of H3K4me2 centered over the ESR1 binding site both before and after ESR1 activation (Fig. 2B, left panels). The pattern of H3K4me2 at the shared AR/FOXA1 and ESR1/FOXA1 sites was also distinct. H3K4me2 signal at the AR/FOXA1 bound sites indicates nucleosome depletion and better positioned flanking nucleosomes after AR activation (Fig. 2A, center panels). In contrast, the pattern at ESR1/FOXA1 sites is similar to the ESR1-unique sites and has a single broad peak both before and after ESR1 activation (Fig. 2B, center panels). NPS, an algorithm that predicts nucleosome position (Zhang et al. 2008b), also predicts clearly different nucleosome distributions relative to ESR1-unique binding sites, FOXA1-unique binding sites, and shared sites (Supplemental Fig. 1C–E). At sites of ESR1 binding with or without FOXA1, the predicted nucleosomes more frequently overlap the ESR1 binding site (Supplemental Fig. 1E,F) while FOXA1 sites that lack ESR1 binding sites have a peak of binding that is in a region removed from a nucleosome center (Supplemental Fig. 1G).

To further test whether the differences between ESR1 and AR are intrinsic to the transcription factors, we examined the MCF-7-derived hormone-independent breast cancer cell line MCF-7:2A (Pink et al. 1995; Ariazi et al. 2011). While MCF-7:2A cells grow in the absence of estrogen or androgen, their growth is inhibited by silencing of either ESR1 or AR (data not shown). Sixty-five percent of the ESR1 binding sites in MCF-7 under the E2-stimulated condition overlap with those of MCF-7:2A in the absence of estrogen (Supplemental Fig. 2C). While there is significant overlap in the ESR1 and AR binding sites in MCF-7:2A, there are also many ESR1- and AR-unique sites (Fig. 2C, Venn diagram). MNase digested H3K4me2 ChIP-seq in MCF-7:2A was performed, and the distribution of H3K4me2 at ESR1-unique, AR-unique, and shared sites was determined (Fig. 2C; Supplemental Fig. 2D). At the ESR1-unique binding sites, H3K4me2 formed a sharp, unimodal peak at the binding site (Fig. 2C, left panel). In contrast, the AR-unique sites are associated with a broader H3K4me2 tag distribution with two modes that flank the AR binding site (Fig. 2C, right panel). Shared ESR1 and AR binding sites had an H3K4me2 profile with an intermediate

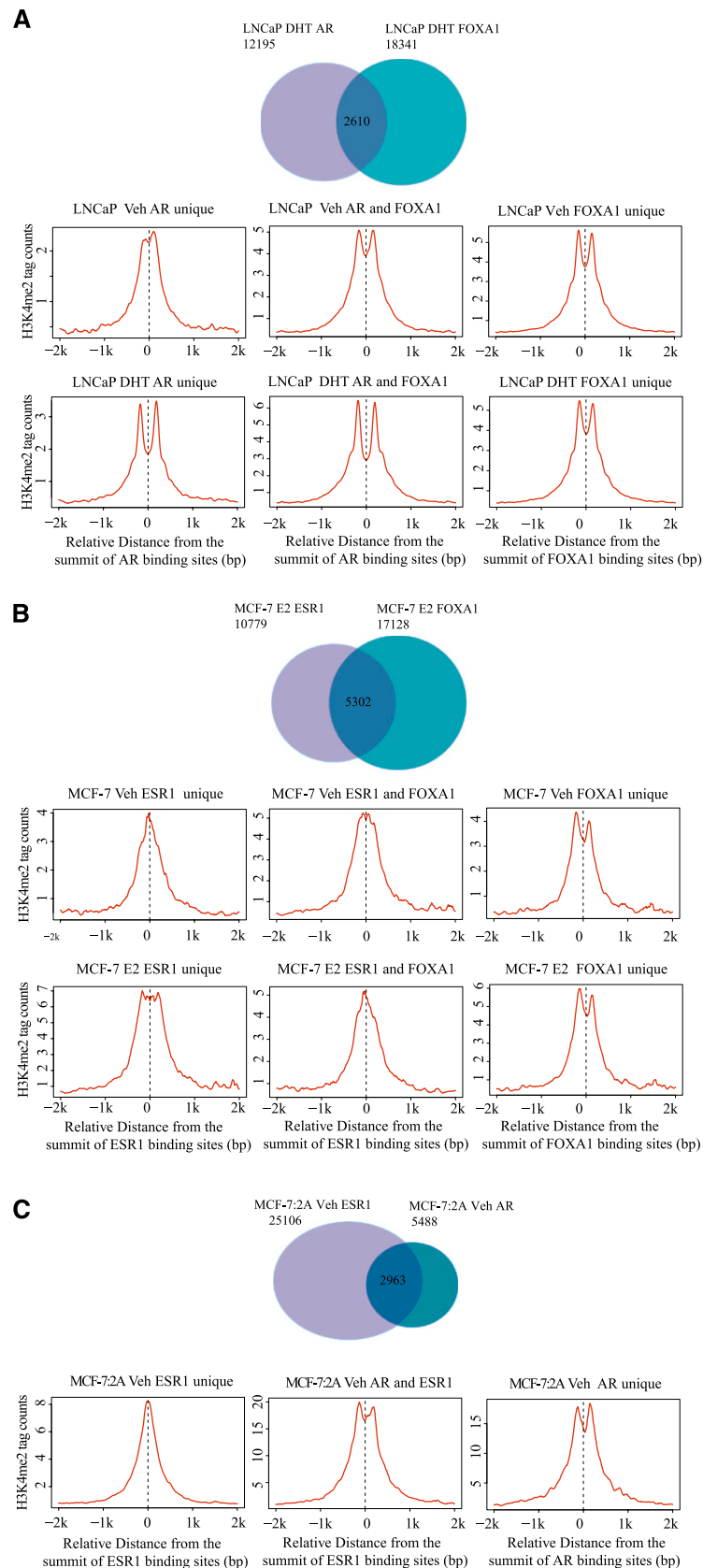


Figure 2. (Legend on next page)

distribution between that of the ESR1-unique and AR-unique sites (Fig. 2C, middle panel). These results suggest that, although AR binding involves depletion of a nucleosome directly over the AR binding site, ESR1 binding does not.

Quantitative measures of DNase I dynamics are predictive of TF binding

Given our finding that ESR1 binding could not be predicted by changes in the occupancy of H3K4me2 marked nucleosomes, we investigated stimulus-dependent changes in DNase I hypersensitivity (DHS) as a complement to nucleosome occupancy. An analysis of DHS under unstimulated (Veh) and androgen-stimulated (DHT) conditions in the LNCaP cell line demonstrated that 51% of AR binding sites overlap with androgen-stimulated DHS regions (Fig. 3A), as would be expected from our prior work on nucleosome occupancy. When we analyzed DHS in MCF-7 cells in unstimulated and estrogen-stimulated (E2) conditions, we found that ~63% of ESR1 binding sites overlap with stimulated DHS regions (Fig. 3B). In LNCaP cells, increasing the sequencing depth from 50 M to 70 M increased the proportion of AR sites that overlapped a DHS site from 51% to 55% (Supplemental Fig. 3A). Similarly, increasing the sequencing depth in MCF-7 cells from 28 M to 70 M raised the proportion of ESR1 sites that overlap with DHS from 63% to 71% (Supplemental Fig. 3B). ESR1 and AR sites that are not associated with DHS show significantly lower levels of binding than those that are associated with DHS (Supplemental Fig. 3C,D).

DHS regions encompass genomic locations that are associated with a variety of transcription factors and other chromatin-associated complexes; therefore, we investigated whether changes in DHS between conditions can be used to enhance the specificity of transcription factor binding site prediction. Starting with the set of DHS regions that were detected under hormone-stimulated conditions, we ranked the regions by three criteria: the DHS tag count in the unstimulated condition, the DHS tag count in the stimulated condition, and a score representing the change in the number of tag counts between the two conditions (Δ DHS) (Fig. 3C,D). The results for the LNCaP AR and MCF-7 ESR1 systems were quite distinct. In LNCaP cells, the level of DHS is not a strong predictor of AR binding in either the unstimulated or stimulated condition, although in both cases it is somewhat informative. In contrast, the change in DHS, Δ DHS, is a very strong predictor of AR binding (Fig. 3C). Interestingly, in the MCF-7 system, the level of DHS under unstimulated conditions is slightly predictive of ESR1 binding; however, estrogen-stimulated DHS and, most significantly, Δ DHS are progressively superior at predicting ESR1 binding (Fig. 3D). These results suggest on a genome-wide scale that at AR and ESR1 binding sites DHS increases upon receptor binding.

On a genomic scale, DNA sequence recognition motifs alone are poor predictors of *in vivo* ESR1 and AR binding. However, within DHS regions, DNA sequence motifs may be useful for iden-

tifying the DHS sites associated with the binding of a particular transcription factor. Starting with the set of DHS regions detected in the hormone-stimulated condition, we ranked the regions by three criteria: Δ DHS, strength of the AR or ESR1 DNA sequence motif, and a combination of the sequence motif and Δ DHS. In both the LNCaP and MCF7 systems, the nuclear receptor binding motifs are capable of discerning the binding locations of the specific factors from the remainder of the open chromatin regions (Supplemental Fig. 4A,B). Therefore, while DNA sequence motifs may not be reliable predictors of transcription factor binding across the entire genome (Carroll et al. 2006), they are reliable predictors within the regions of open chromatin. The best prediction of AR or ESR1 binding, however, was obtained by combining Δ DHS and motif based rankings. To further assess the ability of our approach to predict genome-wide receptor binding sites, we carried out precision-recall analysis for ESR1 (Fig. 3E) and AR (Supplemental Fig. 5). Precision is the fraction of predicted binding sites that are true positives and recall is the fraction of true binding sites identified. As seen for ESR1 in MCF-7 cells, DNA sequence motif alone is a poor predictor of binding. Combining static DHS peaks with motif yields a significantly better prediction, while combining Δ DHS with motif is most predictive. Interestingly when we plotted the precision-recall value for the ESR1 binding sites predicted by the CENTIPEDE algorithm (Pique-Regi et al. 2011) we found a point-prediction (see Methods) that is very similar to what we find using static DHS plus motif. Thus Δ DHS plus motif provides a powerful computational model for TF binding site prediction.

DNase I hypersensitivity is dependent on combinations of bound transcription factors

We further investigated the influence of combinations of ESR1 and AR binding with FOXA1 on Δ DHS. We found that the majority of FOXA1 sites are DHS in the LNCaP (72%) and MCF-7 (64%) cell lines (Supplemental Fig. 6). Interestingly, while DHS tends to increase at shared nuclear receptor FOXA1 sites, FOXA1 sites that do not overlap with AR or ESR1 loci after stimulation are associated with a decrease in DHS (Fig. 3F,G). In addition, Δ DHS at nuclear receptor binding loci are modified by the presence of FOXA1 in a cell line dependent fashion. In MCF-7, ESR1 sites that overlap with FOXA1 loci tend to show larger increases in DHS than the non-FOXA1 binding site containing ESR1 sites (Fig. 3G). In contrast, we observe a larger Δ DHS in non-FOXA1 AR binding sites than in the AR sites that overlap with FOXA1 (Fig. 3F) in LNCaP cells, despite the fact that the hormone-stimulated DHS signals in both cell lines are greatest at the shared nuclear receptor-FOXA1 shared sites (Supplemental Fig. 7).

Coactivator activity is detected by Δ DHS

One motivation for generating cistromes is to gain insight into the regulation of gene expression. To determine if Δ DHS can inform transcriptional regulation, we compared published LNCaP gene expression data (Wang et al. 2009) and MCF-7 GRO-seq data (Hah et al. 2011) with three sets of DHS sites: hormone-increased (top 5000 Δ DHS), hormone-diminished (bottom 5000 Δ DHS), and hormone-unchanged (middle 5000 Δ DHS). In both LNCaP and MCF-7 cells, the ratio of up-regulated genes to non-regulated genes (odds ratio) has a strong positive association with the hormone-increased DHS sites within 20 kb of the transcription start site

Figure 2. Mono-nucleosome level H3K4me2 ChIP-seq at nuclear receptor and FOXA1 binding loci in the MCF-7 (A), LNCaP (B), and MCF-7:2A (C) cell lines. (A) (Top panel) Venn diagram of AR binding in relation to FOXA1 binding. (Middle panel) Distribution of H3K4me2 signal centered on AR-unique, AR/FOXA1 shared, and FOXA1-unique sites in the unstimulated condition. (Bottom panel) Distribution of H3K4me2 signal centered on the AR-unique, AR/FOXA1 shared, and FOXA1-unique sites under conditions of androgen stimulation. (B) (Top panel) Venn diagram of ESR1 binding in relation to FOXA1 binding. (Middle panel) Distribution of H3K4me2 signal centered on ESR1-unique, ESR1/FOXA1 shared and FOXA1-unique sites in unstimulated cells. (Bottom panel) Distribution of H3K4me2 signal centered on ESR1-unique, ESR1/FOXA1 shared, and FOXA1-unique sites in estrogen stimulated cells. (C) (Top panel) Venn diagram of ESR1 binding in relation to AR binding. (Bottom panel) Distribution of H3K4me2 signal centered on ESR1-unique, ESR1/AR shared, and AR-unique sites in unstimulated cells.

(TSS) (Fig. 4A, red bars). In contrast, there is no positive association between hormone-unchanged or -diminished DHS sites with increased gene expression (Fig. 4A, blue and green bars).

We have previously shown using ESR1 ChIP-chip and gene expression microarrays in MCF-7 that early up-regulated genes, which increased after 3 h of hormone stimulation, are strongly associated

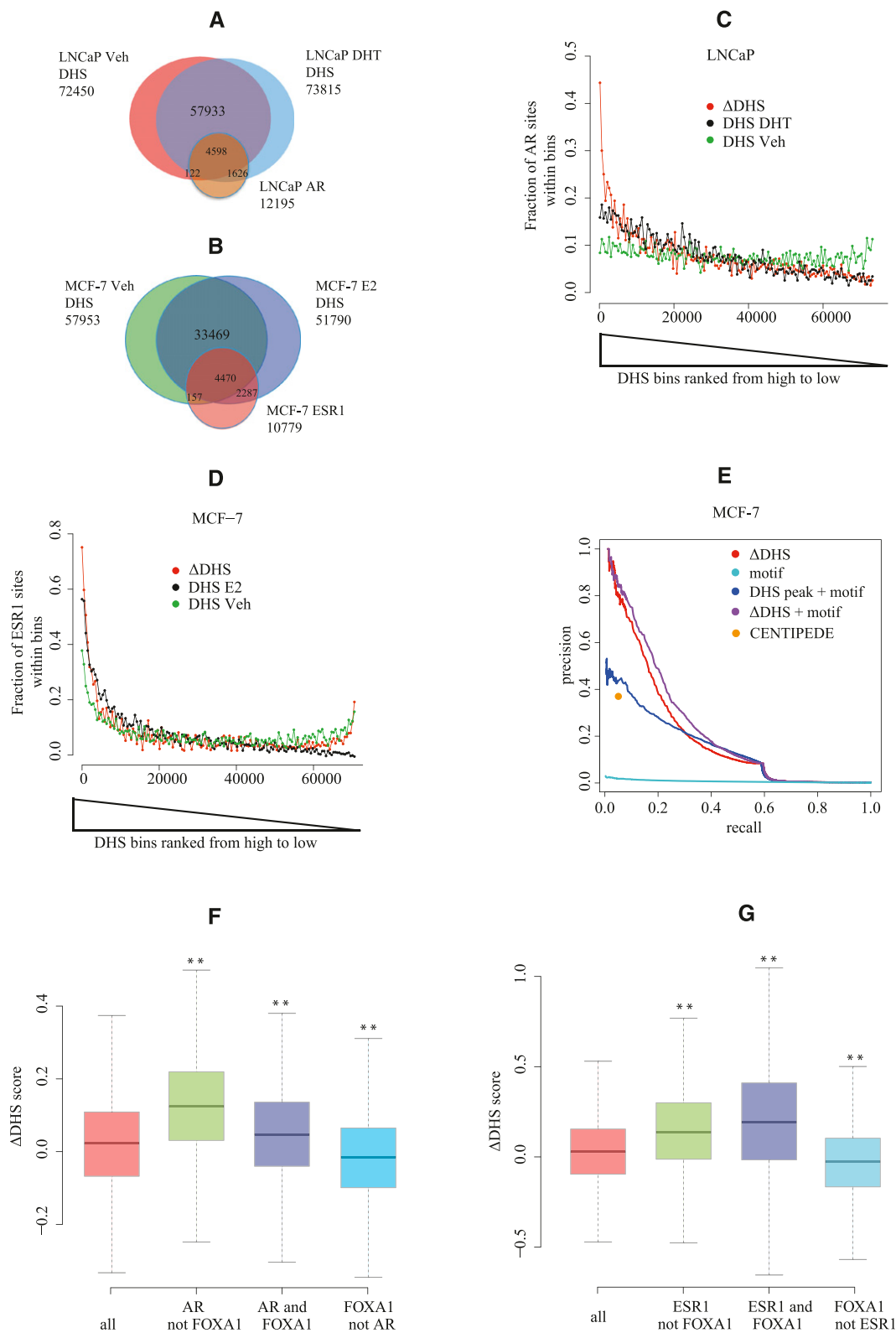


Figure 3. (Legend on next page)

with ESR1 binding, whereas the early down-regulated genes are not (Carroll et al. 2006). These findings were confirmed by Hah and colleagues using GRO-seq (Hah et al. 2011). Interestingly, we find a strong association of early down-regulated genes with the hormone-diminished DHS sites (Fig. 4B, green bars). Motif analysis shows that, while the hormone-induced DHS regions are enriched for motifs for ESR1, forkhead and AP-1, the hormone-diminished DHS sites are enriched primarily for the forkhead motif and not the ESR1 motif (Table 1). We confirmed that FOXA1 binding is enriched at the sites with both the highest and lowest Δ DHS using FOXA1 ChIP-seq data (Fig. 5A). Interestingly, the FOXA1 sites lacking ESR1 are only strongly associated with the sites with the lowest Δ DHS (Fig. 5B). One explanation for these findings would be that, at sites where FOXA1 is bound in the absence of ESR1, FOXA1 binding is reduced upon estrogen stimulation.

To investigate whether FOXA1 sites without ESR1 binding have reduced enrichment upon stimulation, we compared the FOXA1 ChIP-seq reads under vehicle and stimulated conditions (Joseph et al. 2010) within the three categories of 5000 DHS sites (Fig. 5C). Starting with DHS regions detected in the E2-stimulated condition we counted the number of FOXA1 tags obtained from ChIP-seq in unstimulated and E2-stimulated conditions. If we restrict the set of DHS regions to include only the middle 5000 hormone-unchanged regions and plot the FOXA1 tag count for the stimulated condition as a function of that for the unstimulated condition, we see a linear trend, represented by the blue regression line in Figure 5C. In a similar way if we select the top 5000 hormone-increased DHS sites, we again see a linear trend but the slope of the regression line (red) for this trend is greater. This indicates that there is more hormone-stimulated FOXA1 binding in the hormone-increased set than in the hormone-unchanged set. If we select the top 5000 hormone-diminished sites and plot a regression line (green), we see the slope of the regression line through the hormone-diminished set is not significantly lower than that of the hormone-unchanged set. A re-

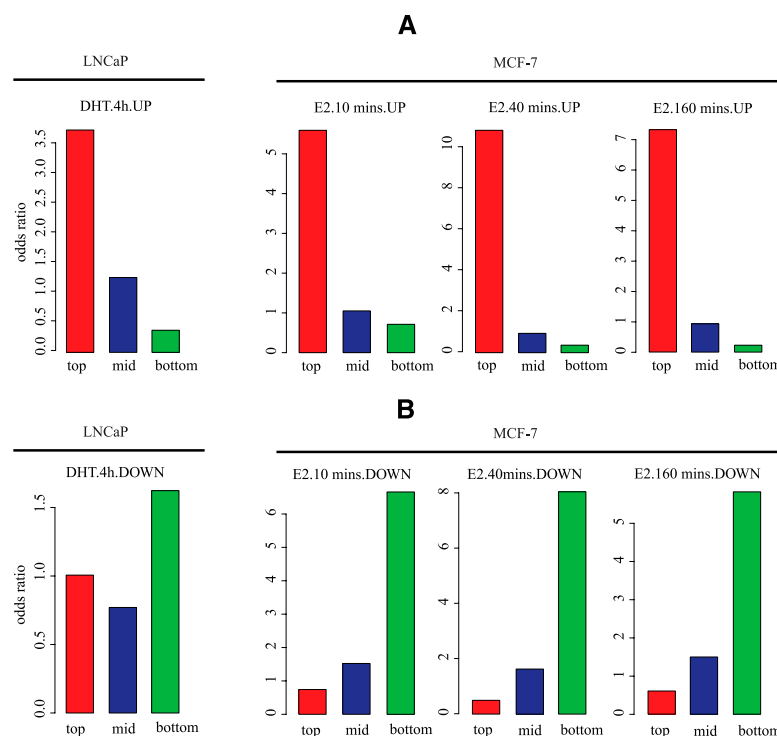


Figure 4. Association between dynamic DNase-seq and differentially expressed genes. Three groups of DHS are represented in LNCaP and MCF-7 cells: hormone-induced DHS sites (red); hormone-unchanged DHS sites (blue); and hormone-diminished DHS sites (green). (Y-axis) Odds ratio calculated by the following formula: (up-regulated genes with at least one nearby site/non-regulated genes with at least one nearby site)/(up-regulated genes with no nearby site/non-regulated genes with no nearby site). In this definition, “nearby” means within 20 kb of the TSS. The hormone-induced sites are associated with up-regulated genes (A), while the hormone-depleted sites are associated with down-regulated genes (B).

duction in FOXA1 binding does not, therefore, appear to explain the decrease in DHS.

Physiological squelching (Meyer et al. 1989) has been postulated to be an important mode of early estrogen down-regulation (Carroll et al. 2006). This phenomenon occurs when multiple factors in the same cell share a common factor, such as a coactivator that is present at a limiting concentration. The transcription factors interfere with each other, “squelching” each other’s influence. Of the numerous known ESR1 coactivators, NCOA3 has been shown to have a particularly strong synergy with ESR1 in enhancing gene expression (Torchia et al. 1997). As with FOXA1, NCOA3 binding was associated with both the highest and lowest Δ DHS sites overall and with only the lowest Δ DHS sites at loci lacking ESR1 (Supplemental Fig. 8).

Figure 3. Characteristics of DNase I hypersensitivity sequencing. (A) Venn diagram of the DHS and AR peaks in LNCaP. The DNase-seq sequencing depth was normalized to the lower sequencing depth for the unstimulated (50 M) and androgen-stimulated (70 M) conditions. (B) Venn diagram of the DHS and ESR1 peaks in MCF-7. DNase-seq sequencing depth was normalized to the lower sequencing depth of the unstimulated (28 M) and estrogen-stimulated (70 M) conditions. (C,D) The fraction of LNCaP AR (C) or MCF-7 ESR1 (D) binding sites in bins ranked by three measures: DNase-seq tag counts in stimulated and unstimulated conditions and a score, Δ DHS, representing the change in DNase I hypersensitivity between the two conditions. The DNase-seq peak regions under the stimulated condition are ranked by these measures. To calculate the proportion of real binding sites as a function of rank, these ranked regions are grouped into bins of 500. (Y-axis) Fraction of regions in each bin that overlap with AR (C) or ESR1 (D) ChIP-seq enriched regions. (E) The precision-recall curves for prediction power of MCF-7 ESR1 binding sites were calculated by five measures: Δ DHS, ESR1 motif, ESR1 motif in E2 DHS, $\sqrt{\text{rank}(\Delta\text{DHS}) \times \text{rank}(\text{motif})}$, and results generated by the CENTIPEDE algorithm on ENCODE MCF-7 DNase-seq data (see Methods). (F,G) Box plots showing the distribution of the DNase-seq change (Δ DHS) between the unstimulated and stimulated conditions in LNCaP (F) and MCF-7 (G) cells. “All” represents all the DHS sites in MCF-7 and LNCaP; “AR not FOXA1” and “ESR1 not FOXA1” represent AR and ESR1 binding sites that do not overlap with FOXA1; “AR and FOXA1” and “ESR1 and FOXA1” represent AR and ESR1 binding sites that overlap with FOXA1; “FOXA1 not AR” and “FOXA1 not ESR1” represent FOXA1 binding sites that do not overlap with AR and ESR1. (**) Wilcoxon rank-sum test P -values < 0.01 , comparing “all” with the other categories.

Table 1. Top 20 motifs enriched in the top 5000 and bottom 5000 MCF-7 Δ DHS regions

Top 5000 Δ DHS				Bottom 5000 Δ DHS			
Motif ID	Gene symbol	Number of hits	P-value	Motif ID	Gene symbol	Number of hits	P-value
M00959	<i>ESR1</i>	2002	1.00×10^{-30}	M00724	<i>FOXA1</i>	3806	1.00×10^{-30}
M00515	<i>PPARG</i>	997	1.00×10^{-30}	M00131	<i>FOXA2</i>	3782	1.00×10^{-30}
M00925	<i>JUN/FOS</i>	1557	1.00×10^{-30}	M01012	<i>FOXM1</i>	4283	1.00×10^{-30}
M00156	<i>RORA</i>	2797	1.00×10^{-30}	M00269	<i>FOXA2</i>	4803	1.00×10^{-30}
M00037	<i>NFE2</i>	1696	1.00×10^{-30}	M00292	<i>FOXD1</i>	3250	1.00×10^{-30}
M00285	<i>NFE2L1</i>	4634	1.00×10^{-30}	M00422	<i>FOXJ2</i>	4171	1.00×10^{-30}
M00495	<i>BACH1</i>	972	1.00×10^{-30}	M00290	<i>FOXF2</i>	4927	1.00×10^{-30}
M00490	<i>BACH2</i>	875	1.00×10^{-30}	M00291	<i>FOXC1</i>	4891	1.00×10^{-30}
M00239	<i>NR1D1</i>	1531	1.00×10^{-30}	M00289	<i>FOXI1</i>	4747	1.00×10^{-30}
M00727	<i>SF1</i>	4133	1.00×10^{-30}	M00266	<i>CROCC</i>	4693	1.00×10^{-30}
M00511	<i>SLC7A1</i>	3291	1.00×10^{-30}	M00268	<i>XFD2</i>	4962	1.00×10^{-30}
M01138	<i>ROR1</i>	3723	1.00×10^{-30}	M01137	<i>FOXO3</i>	4496	1.00×10^{-30}
M00292	<i>FOXD1</i>	4559	1.00×10^{-30}	M00809	<i>FOX factors</i>	3571	1.00×10^{-30}
M00157	<i>ROR2</i>	1568	1.00×10^{-30}	M00472	<i>FOXO4</i>	4543	1.00×10^{-30}
M00204	<i>GCN4</i>	808	1.00×10^{-30}	M00742	<i>FOXJ1</i>	4768	1.00×10^{-30}
M00821	<i>NFE2L2</i>	2809	1.00×10^{-30}	M00267	<i>XFD1</i>	4583	1.00×10^{-30}
M00035	<i>MAF</i>	2896	1.00×10^{-30}	M00951	<i>GRHL3</i>	3561	1.00×10^{-30}
M00269	<i>FOXA2</i>	2247	2.06×10^{-27}	M00294	<i>FOXF1</i>	4859	1.00×10^{-30}
M00724	<i>FOXA1</i>	4452	1.34×10^{-25}	M00475	<i>FOXO3</i>	4259	1.00×10^{-30}
M00983	<i>MAF</i>	949	2.89×10^{-25}	M00473	<i>FOXO1</i>	4800	1.00×10^{-30}

Using published MCF-7 NCOA3 ChIP-seq data (Joseph et al. 2010; Lanz et al. 2010), we compared NCOA3 and FOXA1 cis-tromes, finding 61% of FOXA1 binding sites overlap with NCOA3 (Supplemental Fig. 9). Analyzing the three categories of DHS sites using this NCOA3 ChIP-seq data, we found that NCOA3 binding associated with hormone-diminished DHS loci was distributed in a clearly distinct pattern from the hormone-unchanged sites (Fig. 5D). The slope of the regression line of the hormone-diminished set was significantly lower than that of the hormone-unchanged set (Fig. 5D). As ESR1 directly interacts with NCOA3, these data support the hypothesis that ESR1 competes with FOXA1 for limited amounts of NCOA3 that are either directly associated with FOXA1 or associated with other transcription factors whose binding is facilitated by FOXA1.

If physiological squelching is responsible for the E2-stimulated loss of NCOA3 at FOXA1 binding sites, then higher concentrations of NCOA3 in the nucleus should result in a reduced E2-stimulated NCOA3 loss. We tested this by overexpressing NCOA3 (Supplemental Fig. 10A), selecting six FOXA1 non-ESR1 binding sites from hormone-diminished DHS and determining NCOA3 and FOXA1 binding strength by ChIP-qPCR (Supplemental Fig. 10). The control confirms what we found in the ChIP-seq data: FOXA1 binding does not significantly change on E2 stimulation and there is NCOA3 loss (Fig. 5E). In the NCOA3 overexpression experiment, however, we find no significant change in either FOXA1 or NCOA3 binding on E2 stimulation (Fig. 5F). We also examined the effect of NCOA3 overexpression on the expression of five genes down-regulated by estrogen and found that NCOA3 overexpression reduced the extent of these expression changes (Supplemental Fig. 11). These results are consistent with the physiological squelching mechanism in which E2-induced ESR1 binding sites compete with FOXA1 sites for the NCOA3 coregulator.

Discussion

Using genome-wide DNase-seq and H3K4me2 ChIP-seq analyses, we have mapped important features of enhancer-associated chromatin. We observed systematic differences in nucleosome occupancy

patterns and DHS associated with different transcription factors in LNCaP and MCF-7 cell lines. While AR binding in LNCaP cells has large effects on nucleosome occupancy, ESR1 binding in MCF-7 cells is not strongly influenced by, nor does it influence, nucleosome occupancy. In LNCaP cells, it has been reported that a knockdown of FOXA1 expression causes a dramatic change in AR binding locations, including the gain of numerous sites that are not observed under normal FOXA1 conditions (Wang et al. 2011). Notably, these new AR binding sites were not associated with observable nucleosome remodeling but were more like the ESR1 binding we observed in MCF-7 cells.

Thermodynamic equilibrium has been proposed to explain experimentally observed genome-wide in vivo nucleosome occupancy patterns. In this model, both nucleosomes and transcription factors have an intrinsic affinity for DNA sequence that is dependent on sequence composition (Segal and Widom 2009). Transcription factors compete with nucleosomes for DNA, and thermodynamic equilibrium determines the configuration of nucleosomes and transcription factors. In addition, nucleosome occupancy is likely to be shaped by kinetic elements, in particular, chromatin-remodeling factors using the energy derived from ATP hydrolysis to actively modify DNA-histone interactions. The importance of ATP-dependent factors was demonstrated in a recent study that showed that ATP is essential for creating the strongly positioned nucleosome arrays observed near TSSs in *Saccharomyces* (Zhang et al. 2011). Experimental evidence shows that different chromatin remodeling enzymes are recruited to enhancer loci by sequence-specific transcription factors (Peterson and Workman 2000), such as nuclear receptors. For example, BRG-1, the active component of human SWI/SNF chromatin-remodeling complexes, has been shown to be a key factor that potentiates AR- and ESR1-regulated transcription (DiRenzo et al. 2000; Dai et al. 2008). Both AR and ESR1 are known to interact directly with BAF57, a component of the SWI/SNF remodeling complexes (Belandia et al. 2002; Link et al. 2005). Several modes of chromatin remodeling have been suggested, including nucleosome sliding, nucleosome eviction, and looping of DNA away from the histone core. We speculate that the distinct mechanisms of the different classes

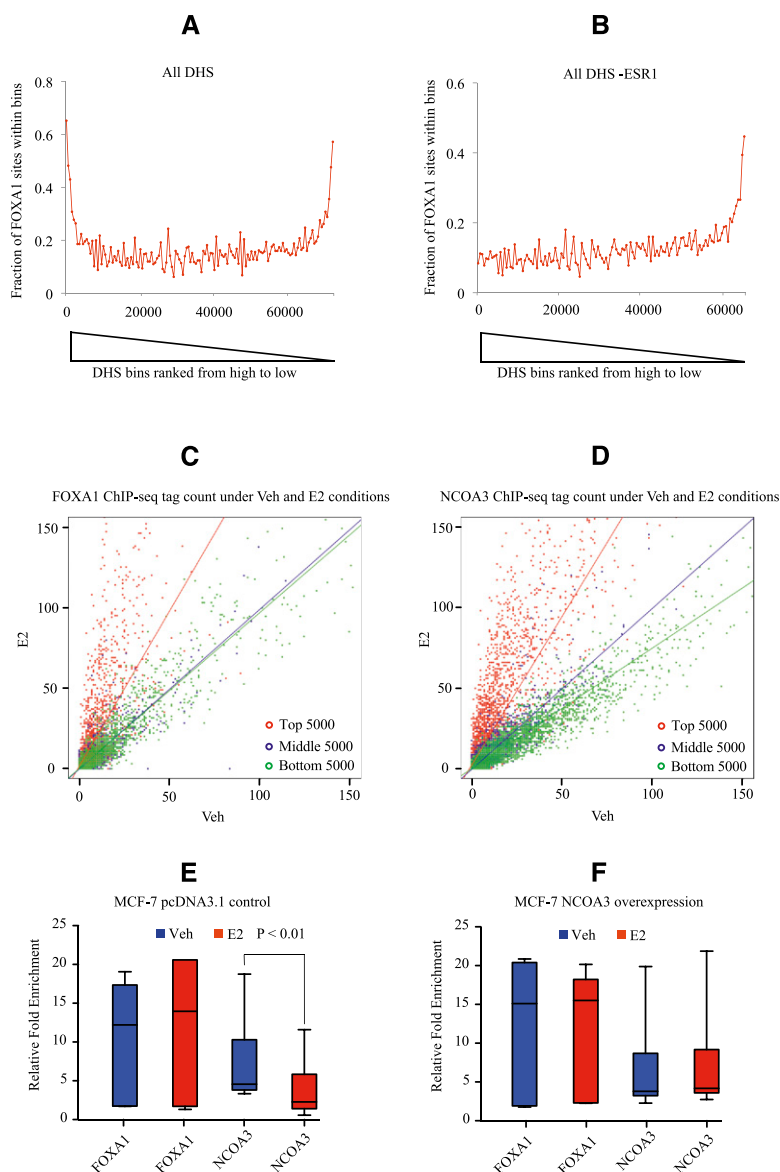


Figure 5. DNase I hypersensitivity changes at FOXA1 and NCOA3 sites. Association of Δ DHS with FOXA1 sites in the presence (A) and absence (B) of ESR1 binding. MCF-7 DHS in the estrogen-stimulated condition were ranked in descending order based on the Δ DHS score. These ranked regions are grouped into bins of 500. (Y-axis) Fraction of regions that overlap with FOXA1 ChIP-seq enriched regions. Scatter plots of FOXA1 (C) and NCOA3 (D) ChIP-seq tag counts in the stimulated condition compared with counts in the unstimulated condition. Three groups of 5000 DHS sites were selected from the MCF-7 estrogen-stimulated DHS sites: DHS-increased (red), DHS-unchanged (blue), and DHS-diminished (green). Regression lines were drawn for each of the groups. The steeper the slope of a regression line, the greater the binding of the factor in the E2-stimulated condition relative to the unstimulated condition. While the slope for FOXA1 in the DHS-diminished category is not significantly different from that in the DHS-unchanged category, the slope for NCOA3 in the DHS-diminished category is less than that for the DHS-unchanged category. This means that within the DHS-diminished category NCOA3 binding tends to decrease on E2 stimulation while FOXA1 binding is maintained at the same level. Changes of FOXA1 and NCOA3 binding strength at FOXA1 binding sites in the overexpression control (E) and NCOA3 overexpression (F) samples under stimulated and unstimulated conditions. Six FOXA1 binding sites were selected from the hormone-diminished DHS sites. Box plots were generated from the ChIP-qPCR data of the six sites tested. The individual ChIP-qPCR assays are shown in Supplemental Figure 10.

of ATP-dependent remodeling enzymes may explain the differential chromatin effects seen in our experiments. Our study demonstrates how MNase digestion and DHS chromatin assays provide complementary information on chromatin structure.

Our differential DNase I hypersensitivity experiments revealed a surprising link between coregulator and chromatin structure. Significantly, this link was not merely a consequence of FOXA1 binding itself. NCOA3 ChIP-seq in MCF-7 cells under vehicle and estrogen-induced conditions revealed that, although a high overlap between NCOA3 and ESR1 was observed, an unexpectedly high overlap between FOXA1 binding sites and NCOA3-enriched loci was also found (Lanz et al. 2010). Previously, coregulators and chromatin remodeling activity had been shown to act synergistically in the AR and ESR1 systems in collaboration with the AR and ESR1 factors themselves (Metivier et al. 2003; Wang et al. 2005). Here, we find evidence for a chromatin remodeling-coregulator synergy that is associated with FOXA1 in the absence of ESR1 or AR. Our experiment supports the hypothesis that physiological squelching is an important mechanism involved in the down-regulation of genes at early time points following estrogen treatment.

According to our current understanding, DNase I hypersensitivity occurs in nucleosome free regions that are close to transcription factor binding sites. Although we do observe many DHS in non-nucleosomal DNA, DHS sites sometimes occur in regions having high nucleosome occupancy. In particular, we identified a set of DHS sites that were associated with ESR1 binding to nucleosomal DNA. The different nucleosome occupancy and DNase I hypersensitivity patterns that we observed are likely dependent on not only the details of the transcription factor-DNA interaction but also on the chromatin environment at the binding site. Relevant aspects of the chromatin environment may include post-translational histone modifications, the composition of the nucleosomes themselves, and the presence of other protein complexes. Histone post-translational modifications may influence transcription factor binding by enhancing the affinity of transcription factor related protein complexes for the modified histone or by reducing the affinity of the histone octamer for DNA. The structure of the nucleosome cores may also determine nucleosomes as being more or less permissive to transcription factor binding as histones that constitute nucleosomes

come in variants, such as H2A.Z, that have been reported to alter nucleosome properties (Jin et al. 2009).

In our analysis of genome-wide dynamic DNase-seq, we noted three important factors that contribute to DNase I hypersensitivity.

First, in agreement with the standard view, the majority of DHS sites occur in nucleosome-free regions. Second, DHS frequently arises as a result of transcription factor binding; however, they do not necessarily occur in nucleosome-free regions. Third, DHS can change with the addition or removal of cofactors. We demonstrated that dynamic DNase-seq is an effective and informative approach that can be used to locate enhancers that regulate a cell's transcriptional response to stimuli.

Methods

Cell line and culture conditions

The prostate cancer cell line LNCaP was obtained from the American Type Culture Collection. LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The hormone-independent breast cancer cell line MCF-7:2A and the parental MCF-7 cell line were from V. Craig Jordan's lab. MCF-7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, $1 \times$ NEAA, and 6 μ g/L insulin. MCF-7:2A cells were maintained in phenol-red-free RPMI 1640 medium supplemented with 10% charcoal stripped FBS. LNCaP and MCF-7 cells were starved in phenol-red-free medium supplemented with 10% charcoal stripped FBS for 3 d before hormone stimulation.

ChIP and ChIP-seq

The ChIP experiments were performed as previously described (He et al. 2010). We used antibodies to ESR1 (Ab-10 from Neomarkers; HC-20 from Santa Cruz), AR (N-20 from Santa Cruz), FOXA1 (ab23738 from Abcam), and H3K4me2 (07-030 from Millipore). Library construction was performed using the Illumina ChIP-seq DNA sample Prep Kit according to the manufacturer's instruction; the libraries were sequenced at a length of 35 bp with the Illumina Genome Analyzer. Model-based Analysis of ChIP-seq (MACS) software (Zhang et al. 2008a) was used to detect ChIP-seq peak regions. Nucleosome Positioning from Sequencing (NPS) software (Zhang et al. 2008b) was used to identify nucleosome positions based on the H3K4me2 ChIP-seq data. Binding Inference from Nucleosome Occupancy Changes (BINOCh) software (Meyer et al. 2011) was used to predict transcription factor binding events from the H3K4me2 NPS data.

DNase hypersensitivity mapping

DNase hypersensitivity mapping was performed as previously described with brief modifications (Ling et al. 2010; John et al. 2011). LNCaP cells were starved for 3 d in phenol-red-free medium supplemented with 10% charcoal stripped FBS and then treated with ethanol or active androgen 5 α -dihydrotestosterone (DHT) at a final concentration of 10 nM for 4 h. MCF-7 cells were starved the same way and then treated with ethanol or 17 β -estradiol (E2) at a final concentration of 10 nM for 45 min. The cells were trypsinized and pelleted prior to washing and resuspension in buffer A (15 mM Tris-Cl [pH 8.0], 15 mM NaCl, 60 mM KCl, 1 mM EDTA [pH 8.0], 0.5 mM EGTA [pH 8.0], 0.5 mM spermidine, and 0.15 mM spermine) to a final concentration of 2×10^6 cells/mL. Nuclei were extracted by adding buffer A containing NP-40. The nuclei were washed with buffer A and resuspended in prewarmed lysis buffer (13.5 mM Tris-HCl [pH 8.0], 87 mM NaCl, 54 mM KCl, 6 mM CaCl₂, 0.9 mM EDTA, 0.45 mM EGTA) at a concentration of 5 M/mL and then digested with different amounts of DNase I (Roche, 0–75

U) for 5 min at 37°C. The reactions were terminated by the addition of an equal volume of stop buffer (1 M Tris-Cl [pH 8.0], 5 M NaCl, 20% SDS, 0.5 M EDTA [pH 8.0], and 10 μ g/mL of RNase A [Roche]) and incubated at 55°C. After 15 min, Proteinase K (final concentration of 20 μ g/mL) was added to each digestion reaction and incubated for 2 h at 55°C. DNA was extracted by careful phenol-chloroform purification. The isolated DNA was run out on a gel, and DNA fragments between 100 and 400 bp long were gel-selected. The libraries were prepared following the Illumina library preparation protocol. DNase-seq libraries were sequenced at the Beijing Genomic Institute and the Center for Cancer Computational Biology (CCCB) at the Dana-Farber Cancer Institute.

NCOA3 overexpression experiments

A total of 12 μ g of pcDNA3.1-NCOA3 construct or the control empty vector were transfected in MCF-7 cells in 10-cm culture dishes using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 72 h of transfection, cells were treated with estrogen or ethanol control for 45 min and then processed for ChIP-qPCR. For RT-qPCR, 3 μ g of the pcDNA3.1-NCOA3 or the empty vector were transfected in MCF-7 cells in six-well plates. After 72 h of transfection, cells were treated with estrogen or ethanol control for 3 h. RNA was isolated using RNeasy mini kit (Qiagen) following the manufacturer's instructions. PCR primers used in this work are listed in Supplemental Table 1.

Model for identifying differential DNase I hypersensitivity locations

DNase I hypersensitive regions were identified using MACS with the default parameters. A tag was considered to belong to a genomic interval if, when shifted 100 bp in a strand-directed direction, the entire tag fell within that interval. Each peak i from the set of m MACS peaks was then given a DHS change score (Δ DHS) by the formula:

$$\Delta DHS_i = \sqrt{n_i^{treat} / \left(\sum_{k=1}^m n_k^{treat} \right) / m} - \sqrt{n_i^{control} / \left(\sum_{k=1}^m n_k^{control} \right) / m}.$$

In this formula, n_i is the tag count in a 600-bp interval centered on the i -th MACS peak. The superscripts *treat* and *control* refer to the hormone-stimulated and vehicle conditions, respectively. We use the square root transformation to stabilize the variance of the score, allowing regions with high counts to be compared with those having low counts. Peaks within 1 kb of any RefSeq TSS were excluded from all analyses so as not to confound transcription factor binding effects with transcriptional ones. All analyses involving motifs enriched in the peak regions were identified using the BINOCh motif analysis software.

Precision recall analysis

To evaluate the ability of our method to predict TF binding we defined a set of bound and unbound genomic locations. We defined the bound set as the summits of MACS peaks determined from ChIP-seq data and located >1 kb from the nearest RefSeq TSS. To define the unbound set, we downloaded a file of "mappable" genomic locations, "wgEncodeCrgMapabilityAlign50mer.bg.gz" from <http://hgdownload.cse.ucsc.edu/goldenPath/hg18/encodeDCC/wgEncodeMapability/> and selected a set of 850,000 non-bound, non-TSS sites by randomly sampling genomic locations that had a mappability index >0.9. These locations were filtered to not lie within 1 kb of any RefSeq TSS, TF ChIP-seq summit or other random location. The background was then scaled up to cover 2 Gb,

the size of the mappable genome. A DHS or ChIP-seq region was considered to be a true positive if its center was within 250 bp of a TF summit and a false positive if its center was within 250 bp of a background site. For motif analysis 200 bp from the center of the DHS or ChIP-seq region was scanned using the BINOCh software (Meyer et al. 2011). CENTIPEDE predictions (Pique-Regi et al. 2011) for ESR1 binding in MCF-7 were downloaded from <http://centipede.uchicago.edu/SimpleMulti/>. In the performance evaluation CENTIPEDE predictions were treated the same way as our DHS regions. Since the result we retrieved from the website contains no scoring information for the sites predicted by CENTIPEDE, a single point was drawn for the performance evaluation.

DHS boxplots

Tag counting under DHS peaks was carried out as before. Peaks were considered to be overlapping if their summits were within 600 bp of each other. Box plots were produced using R with default parameters. The outliers beyond the whiskers are not shown. The *P*-values were calculated using the Mann-Whitney test.

Gene expression data

Affymetrix U133 Plus 2.0 microarray data (GSE7868) (Wang et al. 2007) in LNCaP cells and the processed GRO-seq gene expression data (GSE27463) (Hah et al. 2011) in MCF-7 cells were used in this study. The microarray data were analyzed using the RMA algorithm (Irizarry et al. 2003) using a custom CDF probe (v11) mapping to the RefSeq genes (Dai et al. 2005). The statistical significance was calculated using limma software (Smyth and Speed 2003).

Data access

MCF-7 H3K4me2 ChIP-seq, LNCaP, and MCF-7 DNase-seq raw sequence tags, and processed bed files have been submitted to the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE33216.

Acknowledgments

We thank Dr. David J. Waxman and Dr. Guoyu Ling for help with the DNase-seq protocol. We thank Dr. Jason D. Lieb and Dr. Piotr Mieczkowski for help with the high-throughput sequencing. This work was supported by grants from the National Institutes of Health (1R01 GM099409 to X.S.L.; 2P50 CA090381-06 to C.A.M. and M.B.; 2R01 DK074967-06 to M.B. and X.S.L.; P30 CA051008 to V.C.J.), the Mazzoni Award (GM099409 to X.S.L.), the Department of Defense (W81XWH-10-1-0557 to H.H.H.), and the Prostate Cancer Foundation (to M.B.).

Author contributions: H.H.H., C.A.M., X.S.L., and M.B. designed the experiments and wrote the manuscript. V.C.J. provided the hormone-resistant breast cancer cells and revised the manuscript. H.H.H. and M.W.C. performed the experiments. C.A.M. and H.H.H. conducted the data analysis.

References

Ariazi EA, Cunliffe HE, Lewis-Wambi JS, Slifker MJ, Willis AL, Ramos P, Tapia C, Kim HR, Yerrum S, Sharma CGN, et al. 2011. Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. *Proc Natl Acad Sci* **108**: 18879–18886.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. *Cell* **129**: 823–837.

Belandia B, Orford RL, Hurst HC, Parker MG. 2002. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. *EMBO J* **21**: 4094–4103.

Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoutte J, Shao W, Hestermann EV, Geistlinger TR, et al. 2005. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* **122**: 33–43.

Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoutte J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, et al. 2006. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* **38**: 1289–1297.

Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, et al. 2005. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res* **33**: e175. doi: 10.1093/nar/gni179.

Dai Y, Ngo D, Jacob J, Forman LW, Faller DV. 2008. Prohibitin and the SWI/SNF ATPase subunit BRG1 are required for effective androgen antagonist-mediated transcriptional repression of androgen receptor-regulated genes. *Carcinogenesis* **29**: 1725–1733.

DiRenzo J, Shang Y, Phelan M, Sif S, Myers M, Kingston R, Brown M. 2000. BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. *Mol Cell Biol* **20**: 7541–7549.

Hah N, Danko CG, Core L, Waterfall JJ, Siepel A, Lis JT, Kraus WL. 2011. A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. *Cell* **145**: 622–634.

He HH, Meyer CA, Shin H, Bailey ST, Wei G, Wang Q, Zhang Y, Xu K, Ni M, Lupien M, et al. 2010. Nucleosome dynamics define transcriptional enhancers. *Nat Genet* **42**: 343–347.

Hu G, Schones DE, Cui K, Ybarra R, Northrup D, Tang Q, Gattinoni L, Restifo NP, Huang S, Zhao K. 2011. Regulation of nucleosome landscape and transcription factor targeting at tissue-specific enhancers by BRG1. *Genome Res* **21**: 1650–1658.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Jin C, Zang C, Wei G, Cui K, Peng W, Zhao K, Felsenfeld G. 2009. H3.3/H2A.Z double variant-containing nucleosomes mark ‘nucleosome-free regions’ of active promoters and other regulatory regions. *Nat Genet* **41**: 941–945.

John S, Sabo PJ, Thurman RE, Sung MH, Biddie SC, Johnson TA, Hager GL, Stamatoyannopoulos JA. 2011. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nat Genet* **43**: 264–268.

Joseph R, Orlov YL, Huss M, Sun W, Kong SL, Ukil L, Pan YF, Li G, Lim M, Thomsen JS, et al. 2010. Integrative model of genomic factors for determining binding site selection by estrogen receptor- α . *Mol Syst Biol* **6**: 456. doi: 10.1038/msb.2010.109.

Lanz RB, Bulynko Y, Malovannaya A, Labhart P, Wang L, Li W, Qin J, Harper M, O'Malley BW. 2010. Global characterization of transcriptional impact of the SRC-3 coregulator. *Mol Endocrinol* **24**: 859–872.

Ling G, Sugathan A, Mazor T, Fraenkel E, Waxman DJ. 2010. Unbiased, genome-wide *in vivo* mapping of transcriptional regulatory elements reveals sex differences in chromatin structure associated with sex-specific liver gene expression. *Mol Cell Biol* **30**: 5531–5544.

Link KA, Burd CJ, Williams E, Marshall T, Rosson G, Henry E, Weissman B, Knudsen KE. 2005. BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. *Mol Cell Biol* **25**: 2200–2215.

Lupien M, Eeckhoutte J, Meyer CA, Wang Q, Zhang Y, Li W, Carroll JS, Liu XS, Brown M. 2008. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **132**: 958–970.

Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F. 2003. Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115**: 751–763.

Meyer ME, Gronemeyer H, Turcotte B, Bocquel MT, Tasset D, Chambon P. 1989. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* **57**: 433–442.

Meyer CA, He HH, Brown M, Liu XS. 2011. BINOCh: Binding inference from nucleosome occupancy changes. *Bioinformatics* **27**: 1867–1868.

Peterson CL, Workman JL. 2000. Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr Opin Genet Dev* **10**: 187–192.

Pink JJ, Jiang SY, Fritsch M, Jordan VC. 1995. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* **55**: 2583–2590.

Pique-Regi R, Degner JF, Pai AA, Gaffney DJ, Gilad Y, Pritchard JK. 2011. Accurate inference of transcription factor binding from DNA sequence and chromatin accessibility data. *Genome Res* **21**: 447–455.

Segal E, Widom J. 2009. Poly(dA:dT) tracts: Major determinants of nucleosome organization. *Curr Opin Struct Biol* **19**: 65–71.

- Siersbaek R, Nielsen R, John S, Sung MH, Baek S, Loft A, Hager GL, Mandrup S. 2011. Extensive chromatin remodelling and establishment of transcription factor 'hotspots' during early adipogenesis. *EMBO J* **30**: 1459–1472.
- Smyth GK, Speed T. 2003. Normalization of cDNA microarray data. *Methods* **31**: 265–273.
- Song L, Zhang Z, Gräfeder LL, Boyle AP, Giresi PG, Lee BK, Sheffield NC, Graf S, Huss M, Keefe D, et al. 2011. Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. *Genome Res* **21**: 1757–1767.
- Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG. 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**: 677–684.
- Verzi MP, Shin H, He HH, Sulahian R, Meyer CA, Montgomery RK, Fleet JC, Brown M, Liu XS, Shivdasani RA. 2010. Differentiation-specific histone modifications reveal dynamic chromatin interactions and partners for the intestinal transcription factor CDX2. *Dev Cell* **19**: 713–726.
- Wang Q, Carroll JS, Brown M. 2005. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell* **19**: 631–642.
- Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M. 2007. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol Cell* **27**: 380–392.
- Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, Chen Z, Beroukhi R, Wang H, Lupien M, et al. 2009. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. *Cell* **138**: 245–256.
- Wang D, Garcia-Bassets I, Benner C, Li W, Su X, Zhou Y, Qiu J, Liu W, Kaikkonen MU, Ohgi KA, et al. 2011. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. *Nature* **474**: 390–394.
- Wu C. 1980. The 5' ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. *Nature* **286**: 854–860.
- Zhang Y, Liu T, Meyer CA, Eickhout J, Johnson DS, Bernstein BE, Nussbaum C, Myers RM, Brown M, Li W, et al. 2008a. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**: R137. doi: 10.1186/gb-2008-9-9-r137.
- Zhang Y, Shin H, Song JS, Lei Y, Liu XS. 2008b. Identifying positioned nucleosomes with epigenetic marks in human from ChIP-Seq. *BMC Genomics* **9**: 537. doi: 10.1186/1471-2164-9-537.
- Zhang Z, Wippo CJ, Wal M, Ward E, Korber P, Pugh BF. 2011. A packing mechanism for nucleosome organization reconstituted across a eukaryotic genome. *Science* **332**: 977–980.

Received October 12, 2011; accepted in revised form March 6, 2012.

Jordan, VC. (2012) Estrogen Action, SERMs and Women's Health. World Scientific. New Jersey, London, Singapore (*in preparation*).

Table of Contents

About the Author & Editor

Contributors

Acknowledgements

Foreword

Nancy Brinker

Bert O'Malley

Introduction – Foundation of a Career

V. Craig Jordan

- Chapter 1** **Centennial Article *Cancer Research* 2009. A Century of Deciphering the Control Mechanisms of Sex Steroid Action in Breast and Prostate Cancer: The Origins of Targeted Therapy and Chemoprevention.** *V. Craig Jordan.*
- Chapter 2** **Diethylstilbestrol: The Pioneering Cancer Treatment and Tragedy in Reproductive Endocrinology.** *Joan Lewis-Wambi and V. Craig Jordan.*
- Chapter 3** **Contraception and Women's Health.** *V. Daniel Castracane and V. Craig Jordan.*
- Chapter 4** **Hormone Replacement Therapy and Women's Health.** *Jennifer Eng-Wong, Ifeyinwa Obiorah and V. Craig Jordan.*
- Chapter 5** **Induction of Ovulation by Non-Steroidal Antiestrogens.** *Ifeyinwa Obiorah and V. Craig Jordan.*
- Chapter 6** **Clues About Estrogen and Antiestrogen Action from X-ray Crystallography.** *Geoffrey Greene and V. Craig Jordan.*
- Chapter 7** **Co – Regulator Activity and Their Role in Selective Estrogen Receptor Modulator Action.** *Christopher D. Chien, Anton Wellstein and Anna Riegel.*
- Chapter 8** **Role of Estrogen in Familial Breast Cancer.** *Eliot Rosen and Claudine Isaacs.*

- Chapter 9** **An Advance with Aromatase Inhibitors.** *Saranya Chumsri, Ting Bao, Gauri Sabnis and Angela H. Brodie.*
- Chapter 10** **Adjuvant Antihormone Therapy.** *Anthony Howell and V. Craig Jordan.*
- Chapter 11** **Pharmacogenomics of Tamoxifen.** *Hiltrud Brauch and V. Craig Jordan.*
- Chapter 12** **Antihormone Drug Resistance.** *Robert Clarke and V. Craig Jordan.*
- Chapter 13** **Novel Selective Estrogen Receptor Modulators.** *Surojeet Sengupta and V. Craig Jordan.*
- Chapter 14** **Chemoprevention and Breast Cancer.** *Trevor Powles, Russell E. McDaniel and V. Craig Jordan.*
- Chapter 15** **Selective Androgen Receptor Modulators.** *Christopher C. Coss and James T. Dalton.*
- Chapter 16** **Emerging Principle: Selective Nuclear Receptor Modulators.** *Ping Fan and V. Craig Jordan.*
- Appendix 1** **Scientific Survival Suggestions**
- Appendix 2** **Decades of Discovery**
- Appendix 3** **The “Tamoxifen Team” over a 30-year period: An account of students obtaining a PhD degree (or MD as a medical science researcher in the British system for medical doctors)**

Jordan, VC and Maximov PY (2012) Tamoxifen-Pioneering Medicine in Breast Cancer. Milestones in Drug Therapy. Series Eds: Parnham MJ and Bruinvels J. Springer Basel AG, Basel, Switzerland (*in preparation*).

Table of Contents

- Chapter 1** Discovery and early pharmacology of non-steroidal estrogens and antiestrogens. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 2** Tamoxifen goes forward alone. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 3** Metabolism of tamoxifen: the basis for future drug development. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 4** Adjuvant therapy: the breakthrough in survival. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 5** Selective Estrogen Receptor Modulation (SERM): The Wisconsin story. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 6** Tamoxifen and Carcinogenesis. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 7** Chemoprevention: Cinderella waiting for the ball. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 8** Tamoxifen and raloxifene head to head: the STAR trial. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 9** Evolution of drug resistance to estrogen deprivation. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 10** Estrogen-induced Apoptosis. *V. Craig Jordan and Philipp Y. Maximov.*
- Chapter 11** The legacy of Tamoxifen. *V. Craig Jordan and Philipp Y. Maximov.*

Estrogen Receptor (ER)

Lizie Sweeney¹ and V. Craig Jordan² OBE, PhD, DSc, FMedSci

¹Lombardi Comprehensive Cancer Center, Georgetown University Medical Center

²Scientific Director Lombardi Comprehensive Cancer Center, Vincent T. Lombardi Chair of Translational Cancer Research, Vice Chair of Department of Oncology, Professor of Oncology and Pharmacology, Georgetown University Medical Center

Target

The estrogen receptor (ER) is a nuclear receptor whose primary activating ligand is estrogen. The ER comprises five regions: the activating region, DNA binding domain, hinge domain, ligand-binding domain, and the C-terminus region (Figure 1). There exist two isoforms of ER, ER α and ER β , which are encoded by different genes, on different chromosomes, which have different primary structures. The ER α gene is located on chromosome 6, and encodes a 595 amino acid protein with a molecular mass of about 66 kD (Couse and Korach 1999). The two isoforms also seem to have distinct functions; ER α generally promotes growth while ER β can inhibit growth in some tissues (Deroo and Korach 2006) (Figure 1). The ratio of isoforms differs in tissues and can confer tissue-specific actions of ER (Deroo and Korach 2006). This ER isoform ratio indicates a general trend in growth of tumor cells; that is, a high ER α /ER β ratio is associated with strong proliferation, while the inverse ratio correlates with low levels of cellular proliferation (Deroo and Korach 2006). ER α was the first isoform to be discovered, and is the one to be discussed in this article, as it mediates most of the physiological responses investigated in the laboratory (Couse and Korach 1999).

Biology of the target

Belonging to the nuclear receptor superfamily, the ER resides in the nucleus to bind and retain estrogens that have diffused through the cell from the bloodstream (Jordan 2009). The inactive ER is bound to heat shock proteins that dissociate once the ligand binds, allowing an active conformational change to occur (Couse and Korach 1999). When the estrogen and receptor complex is formed, it binds

to estrogen response elements (EREs), and coregulators are recruited to the ER complex tethered to the promotor region of a target gene. Tissues possess unique levels of the 258 known nuclear receptor coregulators (Jordan and O'Malley 2007). Nuclear receptor coregulator recruitment is considered the rate-limiting factor of transcription in mammals, making the arrival of coactivators and/or corepressors at the **DNA** an integral step in the regulation or control of ER activity (Jordan and O'Malley 2007).

When a ligand binds to the ER, a conformational change occurs to the receptor, depending on the nature of the ligand. X-ray crystallography has offered a detailed look into the structures of ligand-bound ER. An antagonistic or antiestrogenic complex will prop the “jaws” (Helix 12) of the ER open, while a planar steroidal estrogen or estrogenic molecule permit the “jaws” of the receptor to close. Estrogen response elements (EREs) can also influence ER conformation, thereby causing varied recruitment of coregulators and therefore gene function (Jordan and O'Malley 2007).

ER bound with either an estrogen or antiestrogen causes coregulator binding based on the ligand-receptor complex shape. Many varied ligands can bind to the ER, as many varied responses can occur; that is, gradient levels of estrogenicity or antiestrogenicity develop due to recruitment of coactivators or corepressors based on receptor-ligand conformation (Jordan 2008). Figure 1 summarizes the activation of the ER through its signal transduction pathway (Jordan 2006).

Target assessment

The ER was discovered through the injection of [^3H]estradiol into immature rats, followed by analysis of radioactivity in specific tissues. It was found that the uterus and vagina, estrogen target tissues, bound and retained the [^3H]estradiol, while organs such as the kidney and liver, estrogen nontarget tissues, washed out the radioactive marker (Jensen and Jordan 2003). This generated the notion that perhaps a receptor was present in the target tissues, allowing the ligand to induce estrogen-associated cellular function and activity. X-ray crystallography was subsequently used to visualize ligand binding to the purified ER protein (Jensen and Jordan 2003).

Clinically, when a patient presents with breast cancer, a biopsy of the breast tissue is taken and observed under a microscope. The tissue can then be evaluated for the presence and status of the ER by staining with a monoclonal antibody against the ER linked to a fluorescent or radioactive marker.

This immunohistochemistry allows the pathologist or clinician to visualize and quantify the ER level in patients' tumors, a critical step in choosing a treatment process that will be effective for that certain tumor phenotype. If the biopsied cells do not present ER, the tumor is termed ER-negative; if they do, they are termed ER-positive. Immunohistochemistry is also used to evaluate other hormone receptor levels applicable in breast cancer, such as HER2 and progesterone receptor (PR).

Role of the target in cancer

8

High level overview

The ER became the first successful major target for cancer therapy (Jordan 2007), but its role in cancer treatment began as a marker in a diagnostic test to predict whether endocrine ablation, such as oophorectomy, would be of value to the patient (McGuire 1973). The assay indicated whether the patient's tumor was ER-positive, therefore likely responsive to estrogen withdrawal (Deroo and Korach 2006; Jensen and Jordan 2003; Jordan 2009). ER-negative breast cancer tumors do not respond to hormonal therapy because there is no ER present by which cellular functions and replication can be modulated (McGuire 1973).

The ER is considered the first drug target for the treatment of breast cancer. Although physiologic estrogen has been shown to stimulate growth of breast cancer, counterintuitively, high-dose estrogen therapy was successfully used as the first chemical cancer therapy (Haddow et al. 1944). Because the ER has the capacity to be modulated, selective estrogen receptor modulators (SERMs) such as raloxifene and tamoxifen were recognized to be useful in the clinic for targeted ER agonism and antagonism. By targeting the ER with drugs like tamoxifen, patients gain a survival advantage from breast cancer. This ER-directed therapy increases the likelihood of survival, thus demonstrating the efficacy of ER as a major therapeutic target in the breast tumor.

Diagnostic, prognostic, predictive

The ER is used in clinical diagnostics to determine what breast cancer phenotype the patient presents. An ER-positive breast tumor indicates the presence of ER in the tissue, and is one facet used

to describe and diagnose the disease. Further, ER-positive tumors generally represent a better prognosis since it is predicted, reasoned, and demonstrated that ER-positive tumors will respond to hormone therapy.

Therapeutics

Many therapeutics have been proposed and developed based on the mechanism of blocking ER action in breast cancer cells. Since the fate of the ER-mediated gene expression relies on the recruitment of coactivators and/or corepressors, it follows that the ER action can be altered by the presence or absence of coregulators. SERMs catalyze the tissue-specific modulation of the ER (Jordan 2004). SERMs bind to the ER, causing a conformational change in the receptor, thereby influencing what coregulators are recruited to the DNA. This process also depends on the presence and level of coactivators and corepressors in the tissue of interest, and the response elements to which the ligand-bound ER complex binds (Deroo and Korach 2006). Figure 2 details an ideal SERM (Jordan 2004).

SERMs are neither purely antagonists or agonists, but a mixed complex generating partial agonism and partial antagonism when ER-bound. In other words, the conformation the receptor forms when bound to a SERM has mixed affinity for coactivators and corepressors. With that thought, it is logical that concentrations of the coregulators in the physical context of the receptor is of critical importance in determining gene function (Jordan and O'Malley 2007). Tissue-specific SERM actions are not only regulated by coregulators, but also other elements include receptor isoform subtypes, ERE DNA sequences, and the turnover of the ER complex (Jordan and O'Malley 2007) (Figure 1).

Pre-clinical summary

Initial pre-clinical animal studies, as early as 1900, investigated ER knock-out mice to investigate endocrine ablation therapy (Couse and Korach 1999). These studies began to illustrate the effects and actions which require genomic ER function. Other pre-clinical studies illustrated the correlation between breast tumorigenesis and duration of lifetime estrogen exposure (Couse and Korach 1999).

Important present pre-clinical investigation focuses on the resistance that can occur with the current clinical therapy. Acquired resistance is considered to be a major concern that limits the effectiveness of long-term antihormonal therapy. Athymic mouse (immune deficient) studies show that ER-positive PR-positive tumors treated at length with tamoxifen will eventually grow when treated with either estradiol or tamoxifen. Long-term SERM therapy induces a profound change in the signal transduction of breast cancer cells from estrogen-stimulated growth to SERM-stimulated growth (Jordan 2008). After extended antihormone therapy for many years, estrogen, once a breast cancer tumor growth enhancer, remarkably becomes an apoptotic trigger. This clinical and laboratory observation is seemingly counterintuitive, since it is established that oophorectomy can prevent tumors and estrogen can enhance tumor growth in the laboratory (Jordan 2004). The “estrogen paradox” is under intense investigation in the laboratory to facilitate effective translation to clinical practice (Jordan 2008). It had been established in 1944 that high-dose estrogen therapy could cause regression of some breast tumors in post-menopausal patients, a then perplexing paradox (Haddow et al. 1944). This pioneering use of high-dose estrogen, the first clinical therapy to treat any cancer, could not be explained at the time but now supports the principle behind the “estrogen paradox.” In normal physiological pre-menopausal breast cancer environment, the ligand-bound ER promotes tumor growth. When this environment is deprived of estrogen for a prolonged period of time, whether it be through use of SERMs or decades after menopause, drug resistance develops, and estrogen eventually triggers cellular apoptosis in the long-term surviving estrogen-deprived tumor cells (Jordan 2008). Pre-clinical laboratory investigation continues to focus on elucidation of acquired SERM resistance and estrogen-induced apoptosis.

Clinical summary

Though radiation and chemotherapy are also widely used in the clinic, hormonal therapy is the treatment most relevant to the ER. ER-positive breast cancer accounts for about 70% of all breast tumors (Masood 1992). Studies show that estrogen causes growth and proliferation of ER-positive breast cancer cells. Tamoxifen, a SERM, acts as an antagonist of the ER in breast tissue, allowing it to block estrogenic action in breast cancer cells, therefore providing effective therapy. Tamoxifen exhibits

estrogen-like (agonist action) in bone and the uterus (Deroo and Korach 2006). Nevertheless, tamoxifen has had widespread and pioneering success, saving hundreds of thousands of lives by treating breast cancer and becoming the pioneering medicine for the prevention of any cancer (Jordan and O'Malley 2007). Long-term adjuvant therapy targeted the breast ER specifically, and tamoxifen became the first drug approved to successfully treat high-risk pre- and post-menopausal patients. Tamoxifen was also found to inhibit the formation of contralateral primary breast cancer. However, this medicine started life as a failed contraceptive that was reinvented as the “gold standard” for the treatment of breast cancer. Unfortunately, the SERM effect of tamoxifen is evidenced by a small but significant increase in the incidence of endometrial cancer in post-menopausal women. This is an estrogen-like effect in the uterus which limits its use as a chemopreventive for breast cancer in post-menopausal women at high-risk.

In order to carry out its functions, tamoxifen must be converted, by the CYP2D6 enzyme system, to endoxifen. If any component of the enzyme system is mutated or functionally inactivated, tamoxifen resistance can occur. Further, some ER-positive breast cancer cells are intrinsically resistant to tamoxifen, perhaps dependent on the presence or absence of other receptors, such as progesterone receptor (PR) or HER-2/neu (Jordan and O'Malley 2007).

Selective serotonin reuptake inhibitors (SSRIs) are used to lessen menopausal symptoms such as hot flashes that can occur during treatment with tamoxifen. However, paroxetine and fluoxetine, two SSRIs, block tamoxifen's conversion to its active metabolite, endoxifen, thereby nullifying the drug. Fortunately, venlafaxine, a serotonin-norepinephrine reuptake inhibitor (SNRI), does not block CYP2D6 from metabolizing tamoxifen to endoxifen and can be taken simultaneously with tamoxifen to prevent hot flashes (Jordan 2009).

Raloxifene, previously known as keoxifene or LY126758, is another SERM structurally similar to tamoxifen. It began development as a potential breast cancer drug but because of its low bioavailability and cross-resistance with tamoxifen was subsequently found to be better suited for reduction of osteoporosis incidence with the prevention of breast cancer as a beneficial side effect (Jordan 2009). Raloxifene became an effective long-term drug therapy for treatment and prevention of osteoporosis for women at risk with the benefit of reducing the incidence of breast cancer (Cummings et al. 1999). Additionally, raloxifene is available with FDA approval to reduce breast cancer incidence in post-

menopausal women at risk for developing the disease (Vogel et al. 2010). Raloxifene does not increase the incidence of endometrial cancer. A new SERM, lasofoxifene, is 100x more potent than raloxifene for the treatment and prevention of osteoporosis. Its beneficial side effects are a reduction of strokes, breast cancer, endometrial cancer, and coronary heart disease (Cummings et al. 2010).

Other than SERMs, another way that the ER activity can be modified is by limiting the availability of the activating ligand, estrogen. Aromatase inhibitors block the aromatase enzyme either competitively or as suicide inhibitors. This prevents the conversion of androgen to estrogen, therefore blocking estrogen production (Deroo and Korach 2006).

Patients with ER-positive breast cancer respond effectively to treatment with SERMs and aromatase inhibitors; these therapies are used routinely in the clinic. ICI 182,780, also known as fulvestrant, is a pure antiestrogen that enhances ER protein destruction and is used as a second-line therapy after acquired resistance occurs with tamoxifen or aromatase inhibitors (Jordan 2009).

Long-term treatment of ER-positive breast cancer patients with tamoxifen is the standard-of-care for pre-menopausal women. Alternatively, the majority of post-menopausal patients receive aromatase inhibitors instead of tamoxifen since it causes fewer side effects while still preventing estrogenic action (Jordan 2008).

Anticipated high-impact results (bullet points of anticipated data)

- Personalized targeted therapy for ER+ breast tumors
- Elucidation of mechanism of SERM resistance
- Therapy to prevent resistance to SERMs
- Therapy to exploit estrogen-induced apoptosis

The application of the ER as a cancer therapeutic target continues to offer promise in laboratory science and for the benefit of patients worldwide. Past scientific discoveries involving ER modulation have laid the foundation for other hormonal receptors and their applicable cancer therapy and/or prevention. The defining principles drawn from the targeting in breast cancers are already being applied to the androgen receptor (AR) and the treatment of prostate cancer (Chen et al. 2005). In the future, the

therapeutic targeting of the hormone receptor superfamily will have profound impact on cancer medicine.
Investigation continues in this field to optimally exploit the expressed biology in breast tumors.

Cross-references

DNA, PR, AR

(insert Figure1 here)

Figure 1. The potential decision network in estrogen target tissues that could program a ligand receptor complex to activate estrogenic or antiestrogenic responses. There are two distinct estrogen receptors (ERs) (alpha and beta) that are differentially distributed throughout the body. The shape of the ligand can change the shape of the receptor complex. This in turn preprograms the complex to bind either a co-activator or co-repressor protein to enhance the intrinsic activity of the complex for estrogenic responses or reduces intrinsic activity for antiestrogenic responses, respectively. The final decision point is to activate or suppress genes directly at DNA estrogen response elements (ERE) or tether the AP-1 sites to increase gene transcription. Overall, a tissue can modify the decision network through cell surface receptor tyrosine kinases (RTK) enhancing the phosphorylation cascade. This in turn can increase phosphorylation of coactivators or the ER. The balance of decision outcomes modulates the response of a particular tissue.

Reprinted from British Journal of Pharmacology, Volume 147, V. Craig Jordan, Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer, p. S 269-S 276, Copyright (2006), with permission from John Wiley and Sons.

(insert Figure2 here)

Figure 2. Progress toward an ideal SERM. The overall good or bad aspects of administering hormone replacement therapy to postmenopausal women compared with the observed site-specific actions of the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene. The known beneficial or negative actions of SERMs have opened the door for drug discovery to create the ideal SERM or targeted SERMs to either improve quality of life or prevent diseases associated with aging in women.

Reprinted from Cancer Cell, Vol 5, Issue 3, V. Craig Jordan, Selective estrogen receptor modulation: Concept and consequences in cancer, p. 207-213, Copyright (2004), with permission from Elsevier.

References

- Chen J, Kim J, Dalton JT. Discovery and therapeutic promise of selective androgen receptor modulators. *Molecular Interventions* 2005;5:173–88.
- Couse JF, Korach KS. Estrogen receptor null mice: What have we learned and where will they lead us? *Endocrine Reviews* 1999;20(3):358-417.
- Cummings SR, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *Journal of American Medical Association* 1999;281:2189–97.
- Cummings SR, et al. Lasofoxifene in postmenopausal women with osteoporosis. *New England Journal of Medicine* 2010;362:686-696.
- Deroo BJ, Korach KS. Estrogen receptors and human disease. *Journal of Clinical Investigation* 2006;116(3):561-570.
- Haddow A, Watkinson JM, Paterson E. Influence of synthetic oestrogens upon advanced malignant disease. *British Medical Journal* 1944;2:393–8.
- Masood S. Estrogen and progesterone receptors in cytology: a comprehensive review. *Diagnostic Cytopathology* 1992;8:475–491.
- McGuire WL. Estrogen receptors in human breast cancer. *Journal of Clinical Investigation* 1973;52(1):73-77.
- Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. *Clinical Cancer Res* 2003;9:1980–9.
- Jordan VC. Selective estrogen receptor modulation: Concept and consequences in cancer. *Cancer Cell* 2004;5(3):207-213.
- Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *British Journal of Pharmacology* 2006;147(S1):S269-S276.
- Jordan VC. Tamoxifen: Catalyst of the change to targeted therapy. *European Journal of Cancer* 2007;44(1):30-38.
- Jordan VC. The 38th David A. Karnofsky lecture: The paradoxical actions of estrogen in breast cancer -- survival or death? *Journal of Clinical Oncology* 2008;26(18):3073-3082.
- Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: The origins of targeted therapy and chemoprevention. *Cancer Research* 2009;69:1243.
- Jordan VC, O'Malley BW. Selective estrogen receptor modulators and antihormonal resistance in breast cancer. *Journal of Clinical Oncology* 2007;25(36):5815-5824.
- Vogel VG, et al. Update of the national surgical adjuvant breast and bowel project study of tamoxifen and raloxifene (STAR) P-2 trial: Preventing breast cancer. *Cancer Prevention Research* 2010;3:696-706.

Figure 1

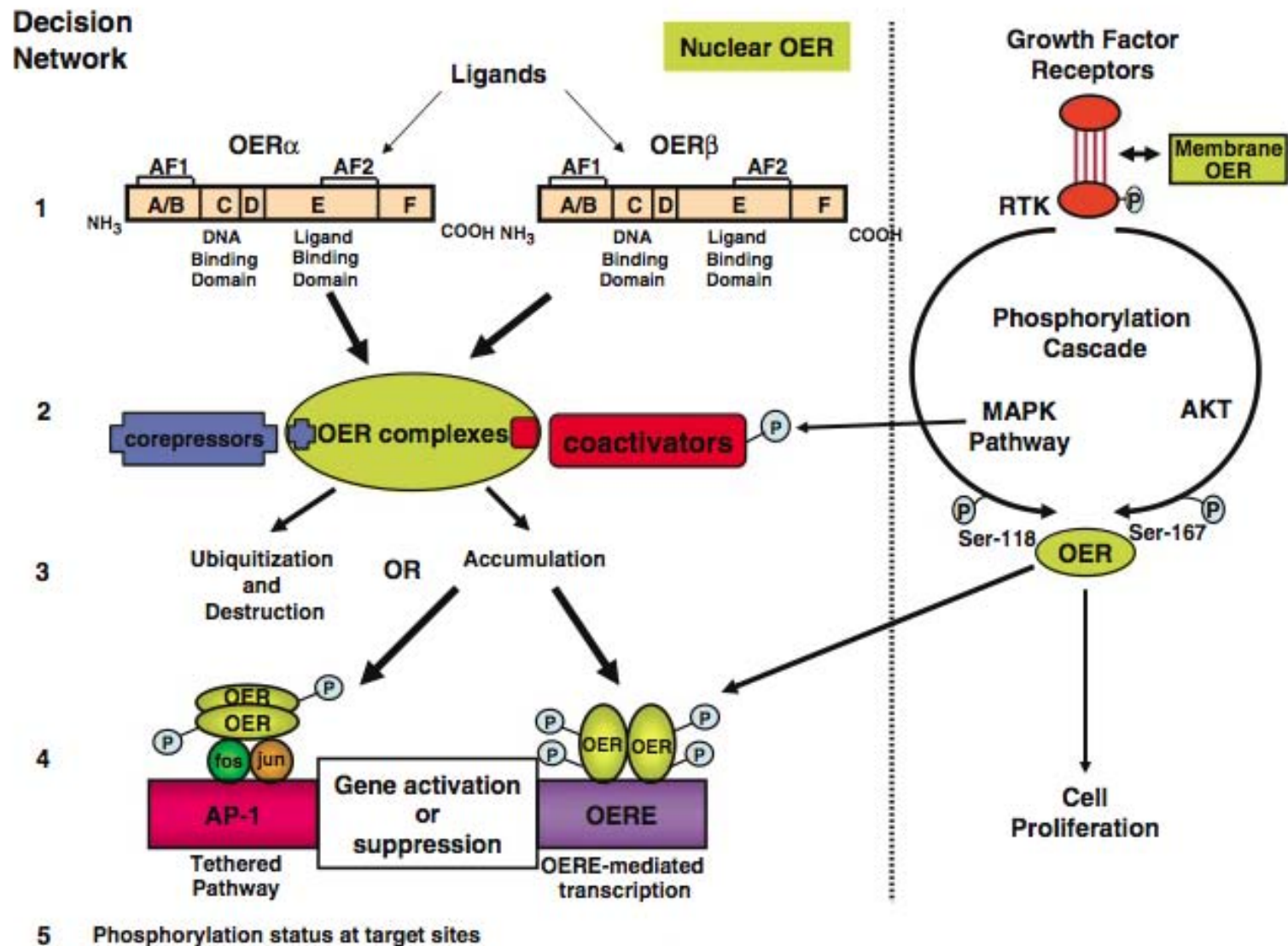
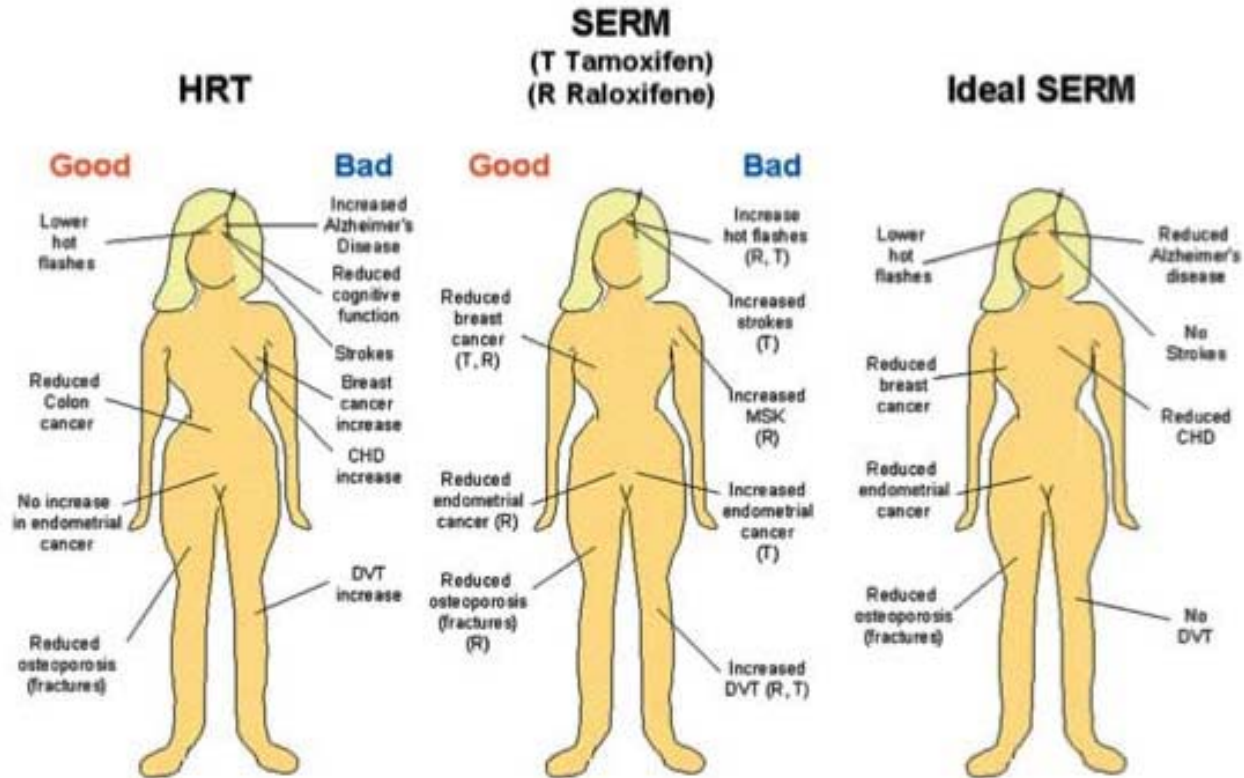


Figure 2



Endocrine Prevention of Breast Cancer

Russell E. McDaniel and V. Craig Jordan

Georgetown Lombardi Comprehensive Cancer Center, Washington DC

Correspondence to:

V. Craig Jordan, OBE, DSc, PhD, FMed Sci

Scientific Director

Georgetown Lombardi Comprehensive Cancer Center

3970 Reservoir Rd., NW

Washington, DC 20057

USA

Introduction

The idea of the prevention of breast cancer is not new, but significant practical progress has been made, through translational research, to make the idea feasible in some women. It is now possible to reduce the incidence of breast cancer through the inhibition of oestrogen action.

Professor Antoine Lacassagne¹ stated a vision for the prevention of breast cancer at the annual meeting of the American Association of Cancer Research in Boston in 1936.

“If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative actions of oestrone, one is led to imagine a therapeutic preventative for subjects predisposed by their heredity to this cancer. It would consist – perhaps in the very near future when the knowledge and use of hormones will be better understood – in the suitable use of a hormone antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breast.”

But no agent that was “antagonistic to prevent the stagnation of oestrone in the breast” was available to the clinician for clinical trial until tamoxifen^{2,3}. Tamoxifen (Fig. 1) became the “antioestrogen” of choice because a) there was a large body of basic biological evidence that this was a valid hypothesis to test b) tamoxifen was noted to reduce the incidence of contralateral breast cancer when used as an adjuvant therapy to treat micrometastases from the original primary tumour and most importantly c) there was a huge and expanding clinical experience with tamoxifen as a long term treatment for node positive and node negative breast cancer. The later point was important as the majority of patients with oestrogen receptor (ER) positive node negative breast cancers are cured by surgery (plus radiation) alone so 5 years of adjuvant tamoxifen was essentially being used in the majority of “well women”^{4,5}.

In this chapter, the changing fashions in endocrine chemoprevention will be described. The change in fashion occurred because of significant advances in our understanding of the pharmacology of the drug group the “nonsteroidal antioestrogens”⁶ that underwent a metamorphosis in the mid 1980’s⁷ to become the new drug group the selective ER modulators

(SERMs)^{8,9}. This laboratory work on SERM action and the finding that antihormone resistance in breast cancer is not static but evolves^{10,11} ultimately led to a discovery (rediscovery?) of a new biology of oestrogen action – oestrogen-induced apoptosis¹². Remarkably, this conversation between the laboratory and the clinical research community now provides a fascinating insight into a paradoxical clinical finding in the Women's Health Initiative (WHI) trial of conjugated equine estrogen (CEE) alone in hysterectomized postmenopausal women in their late 60's. Since dogma dictates that oestradiol is the survival signal that fuels breast cancer cell replication, the WHI trial unexpectedly noted a significant decrease in the incidence of breast cancer during CEE treatment and for the 6 years after treatment stops (cumulative annualized incidence of 151 invasive breast cancers with CEE treatment as opposed to 199 invasive breast cancers with placebo)¹³. These data might provide a starting point for consideration of oestrogen-induced apoptosis as a chemoprevention strategy in the future.

A

The Link between Oestrogen and Breast Cancer

The topic has recently been reviewed¹⁴ in the refereed research literature so only essential facts will be considered here. The link between oestrogen action for breast cancer growth, the original tumour, ER, and 5 years of adjuvant tamoxifen therapy to block tumour growth is compelling and proven in randomized clinical trials¹⁵. The findings can be simply summarized: breast tumours that are ER negative do not respond to tamoxifen treatment, tamoxifen dramatically reduces recurrence and mortality during 5 years of treatment for patients with ER positive breast cancer, and this is maintained for at least 15 years following completion of therapy. Tamoxifen reduces the incidence of contralateral breast cancer by 50% and this is sustained but tamoxifen also increases the incidence of endometrial cancer in postmenopausal women (and mortality). The negative actions of adjuvant tamoxifen, such as deaths from endometrial cancer or thromboembolic disease, do not affect the overall benefit of treatment¹⁵ but do impact on the use of tamoxifen for chemoprevention. Profound target site specific actions of tamoxifen on the uterus in the recent overview¹⁵ recapitulate and confirm the translational research with tamoxifen completed in the 1980's^{16,17} with the recognition of a small but significant increase in the incidence of endometrial cancer in postmenopausal women treated with tamoxifen. This finding eventually resulted in the paradigm shift away from

tamoxifen to new opportunities but this advances our story too quickly. In the 1980's tamoxifen was the only medicine available for testing therapeutic and chemopreventive strategies with SERMs in the 1990's. The clinical community advanced with a responsibility to weigh risks and benefits in clinical trials to ensure the safety and long term health of women at risk for breast cancer.

The treatment trials data base and translational research were essential to address the hypothesis that tamoxifen, a nonsteroidal antioestrogen, could effectively block the genesis and growth of ER positive breast cancer but would be ineffective against the growth of ER negative disease. Nevertheless in the 1980's oestrogen was also considered to be an essential component of women's health by maintaining bone density and preventing coronary heart disease. Thus, if tamoxifen, an antioestrogen, prevented the development and growth of ER positive breast cancer in half a dozen high risk women per year per thousand¹⁸, hundreds of other women in the selected population might subsequently develop osteoporosis and coronary heart disease. The intervention with tamoxifen would be detrimental to public health. The good news was tamoxifen was not an antioestrogen everywhere it was the lead compound of the drug group that selectively modulated ER target tissues around the body. This discovery ultimately facilitated the development of a new strategy for the utilization of new SERMs as chemopreventives in breast cancer.

A

SERM action in the laboratory

The original work to investigate the target site pharmacology of tamoxifen in the laboratory was to provide a database with which to predict clinical outcomes and safety for future chemoprevention trials. Historically in the 1960's there was general interest in the chance finding that nonsteroidal antioestrogens lowered circulating cholesterol. Unfortunately severe toxicological findings were an issue for some compounds because of their ability to increase the level of circulatory desmosterol, which was associated with cataract formation. This toxicity made a search for safer antioestrogens imperative². The discovery of ICI 46,474 (Fig. 1), the pure trans isomer of the substituted triphenylethylene that was to become tamoxifen, was notable because there was a low conversion to desmosterol though circulating

cholesterol was lowered profoundly in rats¹⁹. Indeed the first patent application for tamoxifen in the U.K. stated² in 1965,

“The alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone-dependent tumours or for the management of the sexual cycle and aberrations thereof. They also have useful hypocholesterolaemic activity”.

However, the patent was denied in the U.S. and the statements concerning breast cancer had to be removed initially as the claim was considered to be “fantastic” and without experimental evidence. The patent for tamoxifen in the United States was finally awarded in 1986 just at the time that the National Cancer Institute recommended adjuvant tamoxifen as the standard of care for patients with ER positive breast cancer²⁰.

Parenthetically, all studies conducted in my laboratory during the 1970’s and 1980’s on the application of tamoxifen for the treatment and prevention of breast cancer in the U.S. and England were at a time of no patent protection in the U.S. No other company exploited the findings as no one cared because it was unlikely to be a successful therapeutic strategy!

During the 1980s the Wisconsin Tamoxifen study followed up the question of tamoxifen treatment lowering circulating cholesterol in postmenopausal patients^{21, 22} and noted a decrease in low density lipoprotein cholesterol but no effect on high density lipoprotein cholesterol. There was certainly some initial enthusiasm that there would be a significant decrease in coronary heart disease but despite some encouraging reports²³⁻²⁵ no consistent decrease in coronary events has been noted in the Oxford Overview Analysis for tamoxifen treatment.

Tamoxifen maintains bone density in ovariectomized rats²⁶⁻²⁸ and this counterintuitive laboratory result for an “antioestrogen” formed the scientific basis for the Wisconsin Tamoxifen Study. The clinical study was a placebo controlled double blind trial to establish the actions of 2 years tamoxifen on bone density in post-menopausal patients with node negative breast cancer (at the time of recruitment, these patients were several years post diagnosis and surgery and no adjuvant treatment was the standard of care). Tamoxifen significantly improved bone density compared to placebo treatment²⁹.

Thus tamoxifen was oestrogen-like, lowering circulating cholesterol and oestrogen-like, maintaining bone density so tamoxifen might provide benefit for women enrolled in a chemoprevention trial. The anticancer actions of tamoxifen were well established and supported by the inhibition of mammary carcinogenesis in rat^{30,31} and mouse³² models. But an increase in the incidence in endometrial cancer was a predictable concern, based on earlier work^{16,17} before major clinical trials of chemoprevention in breast cancer started. Also the finding that tamoxifen was a hepatocarcinogen in specific rat strains³³ was of significance toxicologically for safety reasons in any chemopreventive trials, however, it is fair to say that no evidence either at that time^{34,35} or subsequently has demonstrated hepatocarcinogenesis in humans with tamoxifen.

The first pilot chemoprevention study was initiated by Trevor Powles at the Royal Marsden Hospital in the early 1980's³⁶. This study grew over the years of accrual and interestingly showed benefit at 20 years for those women taking tamoxifen for 8 years following recruitment³⁷. However, the pivotal chemoprevention study was the Fisher P-1 study (Fig. 2) conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP)³⁸. This landmark study was an adequately powered prospective, placebo controlled trial primarily used by the Food and Drug Administration as evidence to approve tamoxifen for the reduction of risk of breast cancer in pre and post-menopausal women at high risk for the disease. `

There are significant benefits for women at risk for breast cancer nested within the results of the P-1 prevention trial during treatment with tamoxifen. There were fewer fractures but this was not significant overall. Tamoxifen reduces ER positive invasive breast cancer incidence by 50% and the same is true for ductal carcinoma *in situ* (DCIS)³⁸. Benefits in breast chemoprevention last for years following the cessation of treatment³⁹ and this has been confirmed by others⁴⁰. This is clearly a consistent long term “antitumour action” of tamoxifen imprinted following therapy as noted by the sustained antitumour effect of tamoxifen following adjuvant treatment^{15,41}. We will comment further on the new concept of “imprinting” further in the **SERM summary**.

Despite extensive testing, tamoxifen is seen as presenting the well woman with significant risks such as endometrial cancer and blood clots (it must be stressed only in postmenopausal women)³⁸(Fig.3). There is also the

nagging concern about rat hepatocarcinoma. Tamoxifen has a human carcinogen black box designation in the U.S. With all these uncertainties clearly another strategy for chemoprevention was necessary for an appropriate science based advance in public health. This was obvious⁷ even before the NSABP trial had been launched in the early 1990's³⁸ but tamoxifen was the only medicine available with sufficient clinical trials experience to move forward into chemoprevention. Nevertheless, the recognition of SERMs in the laboratory⁷ also catalyzed a change in the development of another nonsteroidal antioestrogen keoxifene (Fig. 1). Keoxifene was initially investigated in the 1980s as a competitor for tamoxifen as a breast cancer drug but which failed to advance in development as it failed in clinical trial⁴². Surprisingly, keoxifene also maintained bone density in rats similar to tamoxifen but was significantly less uterotrophic than tamoxifen^{26,43}. This would later translate to a reduced risk of endometrial cancer in all subsequent clinical trials. The name was changed from keoxifene to raloxifene (Fig. 1).

Keoxifene prevented mammary cancer in rats but because of poor pharmacokinetics and rapid excretion keoxifene does not have the sustained actions of tamoxifen³¹. Continuous therapy was necessary. Thus the scene was set for a move away from a broad therapeutic strategy with tamoxifen administered to high risk populations where a few ER positive invasive breast cancers can be prevented but the majority of women are exposed to side effects with no benefit to balance the risks. In response, a “roadmap” was created based on laboratory science and the emerging clinical trial data that would significantly advance women’s health.

A

A plan to use SERMS to prevent multiple diseases in women

A plan to prevent breast cancer as a public health initiative was initially described at the First International Chemoprevention meeting in New York in 1987⁴⁴. It is reasonable simply to state the proposal, published from the 1987 meeting⁴⁴ and subsequently refined and presented again at the annual meeting of the American Association for Cancer Research in San Francisco in 1989⁷. *“The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused on the identification of a population of high-risk women to test “chemopreventive” agents. But, are resources being used less than optimally? An alternative would be to seize on the developing*

clues provided by an extensive clinical investigation of available antioestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, then a majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain anti-breast tumour actions, then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone-dependent disease to hormone-independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century⁴⁴". The vision of the concept was refined and focused by 1990⁷. "We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so apparently, derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer." This concept is exactly what has been translated to clinical practice^{45, 46}: use a SERM (raloxifene) to treat osteoporosis and reduce the incidence of breast cancer as a beneficial side effect⁴⁵⁻⁴⁷.

A

The SERMs surface in clinical practice

Raloxifene is the pioneering SERM approved for the prevention of osteoporosis around the world. The pivotal registration trial was the Multiple Outcomes of Raloxifene Evaluation (MORE) trial. Raloxifene reduced spine fractures by 50% compared to placebo⁴⁷. A separate analysis of breast cancer incidence demonstrated a 76% decrease in the incidence of invasive positive breast cancer (Fig. 4) over the 3 year evaluation. There was no increase in endometrial cancer but DCIS remained unaffected⁴⁵. A long running trial, Raloxifene Use for the Heart (RUTH), to examine whether coronary heart events could be reduced in high risk populations, did not slow benefit for raloxifene⁴⁸. Looked at

another way, it showed little harm, but coronary heart disease (CHD) in a high risk population was unaffected.

The use of oestrogen-like medicines to treat and prevent osteoporosis in the postmenopausal woman demands long term therapy – perhaps indefinite therapy. The extension trial to MORE was Continuing Outcomes Relevant to Evista (CORE)⁴⁶. An evaluation of both breast cancer and endometrial cancer in the CORE trial confirmed a sustained efficacy to prevent the development of breast cancer over the 9 years of raloxifene treatment (Fig. 5) and this effect was entirely expressed in the prevention of ER positive disease with no effect on the development of ER negative disease.

Not unexpectedly the promising data from the MORE trial⁴⁵ would propel raloxifene into a head to head study of tamoxifen and raloxifene (STAR) in high risk postmenopausal women (Fig. 6). The STAR trial teaches several important lessons. However the dramatic decrease in invasive breast cancer noted in the MORE trial (raloxifene reducing the risk of ER positive breast cancer by 90% - and a 76% reduction of any newly diagnosed invasive breast cancer)⁴⁵ was not noted in STAR with raloxifene. There was no difference between the incidence of breast cancer during treatment with tamoxifen or raloxifene⁴⁹ notwithstanding the presumed 50% decrease based on the results from the P-1 trial^{38, 39}. Raloxifene had a very low proliferative effect on the uterine epithelium when compared with tamoxifen and this translated to fewer hysterectomies in the raloxifene treated women⁴⁹. Additionally there were fewer thrombotic events with raloxifene and fewer operations for cataracts (see earlier concerns with the triphenylethylene based nonsteroidal antioestrogens²). Overall raloxifene seems to be equivalent to tamoxifen as a chemopreventive for invasive breast cancer but raloxifene is less effective than tamoxifen at controlling the development of DCIS. Nevertheless, raloxifene confers greater safety.

However, the importance of long term follow up for clinical trials is illustrated by STAR. A recent evaluation of the STAR trial 3 years after stopping the 5 years of treatment showed that although tamoxifen retained its “imprinting” as an antitumour agent raloxifene did not. Raloxifene was only 78% as effective at reducing primary breast cancer incidence as

tamoxifen. These clinical data reflect the superiority of tamoxifen in preclinical studies^{31, 50} and based on the raloxifene extension study, raloxifene⁴⁶ may need to be given indefinitely to prevent both osteoporosis and breast cancer.

A

Recent innovations in SERM development

The story of the initial discovery and clinical applications of the SERMs tamoxifen and raloxifene is one of the play of chance with the right people being in the right place at the right time and the willingness to seize an opportunity that ultimately results in progress in medicine and pharmaceutical profits. The profits have to occur to permit progress in medicine. This is not a new idea as it was stated as being essential by Professor Paul Ehrlich in the final days of the 19th century for the successful development of the first chemical therapy (chemotherapy) for any disease (syphilis)⁵¹. Salvarsan (606) was discovered through systematic organic synthesis and testing of hundreds of compounds in appropriate animal models of human disease. But syphilis would not have been conquered if the pharmaceutical company Hoechst had not developed the drug. Without successful drug development, there would be no medicines. This fact is critical to the next part of the SERM story.

There has been considerable innovation by pharmaceutical chemists to refine the selectivity of SERMs and advance in the creation of the ideal SERM. The goal is illustrated in Figure 7. Numerous compounds have been synthesized and tested in preclinical studies but it is not our intention to survey progress in the laboratory here. This progress has been documented elsewhere^{52, 53}. Rather, four SERMs are selected for consideration: ospemifene, arzoxifene, bazedoxifene, and lasofoxifene (Fig. 8). The reason for the selection of these four is that significant progress has been made in completed clinical trials

B

Ospemifene

Ospemifene (FC-1271a) is a new SERM that has shown oestrogen-like effects in bone marrow⁵⁴, enhancing osteoblast formation *in vitro* by a mechanism unlike that of raloxifene. Ospemifene, Z-2-(4-(4-chloro-1,2-diphenyl-but-1-enyl)phenoxy) ethanol) is a metabolite of toremefene⁵⁵. Ospemifene also has oestrogenic activity in the vaginal epithelium, though

not in the endometrium, suggesting its application as a treatment for vaginal dryness associated with menopause^{56, 57}. Ospemifene has been shown to inhibit the growth of the ER positive MCF-7 cells in culture⁵⁶

Preclinical studies *in vivo* have shown ospemifene to prevent bone loss and increase bone strength in ovariectomised rats and to have a benefit in lowering serum cholesterol levels⁵⁸.

Phase I, II, and III clinical trials have been carried out with ospemifene⁵⁵ with no toxicity shown. Phase II trials^{56, 59} and a phase III trial⁶⁰ indicate that ospemifene is effective for treating vulvar and vaginal atrophy in postmenopausal women. Ospemifene's oestrogen-like activity on the vagina improved symptoms of vaginal dryness, unlike raloxifene⁶¹.

B

Arzoxifene

Arzoxifene (LY353381) is a potent SERM that was evaluated by Eli Lilly and Company⁶². This SERM binds to the oestrogen receptor alpha with higher affinity than raloxifene⁶²⁻⁶⁴. It was found to have antagonistic effects on the uterus whilst being 30 to 100 times more potent than raloxifene in the prevention of body weight, bone, and serum cholesterol changes brought about by ovariectomizing rats⁶⁵. Furthermore, arzoxifene and its metabolite, demethylated arzoxifene, have been shown to not have a proliferative effect on endometrial tissue while protecting bone.

In clinical trials, arzoxifene has shown promise for treatment of osteoporosis. In a phase III trial⁶⁶, arzoxifene treatment of postmenopausal osteoporotic women increased spine and hip bone density. Other trials have suggested that arzoxifene was effective against vertebral fractures but not non-vertebral fractures.

In spite of arzoxifene's encouraging preclinical and early clinical findings, arzoxifene is not on the market and is not being developed. Arzoxifene has some adverse effects in common with all SERMs such as hot flashes, increased risk of venous thromboembolic events, and cramps. In addition, a phase III breast cancer clinical trial was stopped because "*Arzoxifene was statistically significantly inferior to tamoxifen with regard to progression-free survival and other time-to-event parameters, although tumor response was comparable between the treatments*".⁶⁷ Arzoxifene has not been developed further.

Bazedoxifene, a SERM for the treatment and prevention of osteoporosis in postmenopausal women (as well as, in combination with conjugated equine oestrogens, for treatment of menopausal symptoms⁶⁸), is currently approved for use in the European Union; and, it is under review by the United States' Food and Drug Administration. This SERM, developed by collaborative efforts between Wyeth Pharmaceuticals and Ligand Pharmaceuticals, has a binding affinity for the ER α about 10-fold lower than 17 β -oestradiol^{69 70}. Preclinical studies on basedoxifene have been two-tiered: those studying basedoxifene alone as treatment and a preventative agent for osteoporosis and those of basedoxifene in combination with conjugated oestrogens. Bazedoxifene alone shows its efficacy in maintaining bone mass in doses as low as 0.1mg/kg/day in ovariectomized rats^{69, 70}. This bone preservation is comparable to raloxifene and lasofoxifene^{71, 72}.

Combination studies have been carried out on basedoxifene given with a mixture of the 10 principal conjugated oestrogens (CEs) in Premarin. Bazedoxifene (3.0mg/kg) was given in tandem with the CE. The basedoxifene antagonized CE induced dose-dependent, increase in uterine weight to control levels⁷³. Another study compared raloxifene, basedoxifene, and lasofoxifene to conjugated oestrogens. Bazedoxifene was unique amongst raloxifene and lasofoxifene in its ability to antagonize effect of CE on uterine weight⁷⁴.

Bazedoxifene has gone through several phase III clinical trials. It has been shown to reduce bone turnover and to prevent bone loss without undue endometrial, ovarian and breast risks^{75, 76}. Another phase III study showed that basedoxifene reduced the incidence of vertebral fractures as compared to placebo⁷⁷. Amongst high risk women, basedoxifene significantly lowered the risk of nonvertebral fracture relative to both placebo and raloxifene⁷⁷. Bazedoxifene is considered to be well tolerated; serious adverse events and discontinuations are similar to those of a placebo group⁷⁸. Any increased risks of venous thromboembolism are similar to raloxifene and lasofoxifene^{79, 80}. Bazedoxifene is considered safe regarding osteoporosis treatment and prevention⁷⁸; but cannot be considered a chemopreventive for breast cancer^{77, 78}.

Lasofoxifene

Lasofoxifene is a SERM which binds with high affinity to the ER that is approved for the treatment of osteoporosis in the U.K. and the E.U. but not currently in the U.S.⁸¹. Animal model studies of lasofoxifene have shown it to inhibit osteoclastogenesis, prevent bone loss and reduce bone turnover^{72, 82}.

Phase II and phase III clinical studies have confirmed improvements in bone mineral density (BMD). In one phase II study⁸³ one year's treatment with lasofoxifene showed significant improvement regarding lumbar spine BMD as compared to calcium and vitamin D. In another study, lasofoxifene acted positively by increasing BMD comparably to CEE⁸⁴.

Three phase III clinical studies have been carried out on lasofoxifene: The Postmenopausal Evaluation and Risk-Reduction with Lasofoxifene (PEARL) study, The Osteoporosis Prevention and Lipid Lowering (OPAL) study, and the Comparison of Raloxifene and Lasofoxifene (CORAL) study. The PEARL study found that both lumbar spine and femoral neck BMD were increased after three years treatment. Lasofoxifene also significantly reduced the risk of ER positive breast cancer as compared to placebo⁸⁵⁻⁸⁷. The OPAL trial tested three doses of lasofoxifene against placebo. All doses showed improved lumbar spine and hip BMD as compared to placebo⁸⁸. All doses also showed reduced serum levels of C-terminal telopeptide of type 1 collagen, serum osteocalcin, no increase in breast density or pain⁸⁹. The CORAL study found that lasofoxifene maintained BMD in the lumbar spine better than raloxifene, with no difference in hip BMD; lasofoxifene also lowered total cholesterol more than raloxifene⁹⁰.

Lasofoxifene is a major advance towards improved potency and side effect profile. Lasofoxifene is 100 times more potent than raloxifene and unlike raloxifene, lasofoxifene reduces the risk of coronary heart disease and strokes. Like raloxifene, lasofoxifene reduces the incidence of fractures and ER positive breast cancers with no increase in the risk of endometrial cancer^{80, 87}.

SERM summary

The practical application of SERMs for the chemoprevention of breast cancer is one that has only resulted from the research philosophy first defined by Professor Paul Ehrlich to achieve successful outcomes in experimental therapeutics, *ie*: the 4 G's – Gluck (luck), Geduld (patience), Geshick (skill) and Geld (money)⁵¹. The discoveries with both tamoxifen and raloxifene, in the same laboratory, were not predictable. Some would say lucky. But with patience and skill over decades and the investment of money from philanthropy and the pharmaceutical industry to develop the new concepts further and “sell” the idea to physicians, millions of women are alive and millions more continue to benefit. The approved drugs, tamoxifen and raloxifene, are safe and effective if used in the correct manner for the right patients: tamoxifen for 5 years in the high-risk premenopausal woman (or the postmenopausal woman without a uterus) or raloxifene indefinitely in the high-risk postmenopausal woman. This therapeutic intervention will reduce the incidence of breast cancer in select populations. By contrast, forty years ago there was nothing.

Unfortunately to advance further, it is unclear whether the new SERMs have the “uniqueness” to overcome raloxifene as the SERM of choice to prevent both osteoporosis and significantly reduce the risk of breast cancer. Arzoxifene can be viewed as a “long acting raloxifene” but following the completion of clinical trials the decision was made not to seek Food and Drug Administration (F.D.A.) approval. In contrast bazedoxifene for osteoporosis or bazedoxifene plus CEE to treat menopausal symptoms appears to have merit for the postmenopausal women with a uterus. Basedoxifene is approved in several countries. Ospemifene could have a “niche” application to ameliorate vaginal dryness, but an application to prevent breast cancer, like toremifene before, is unlikely without major clinical trials for chemoprevention, osteoporosis or other indications. Lasofoxifene is approved in the E.U. but the drug has not been launched. The F.D.A. has not approved lasofoxifene. It is all about Geld and the fear of financial failure by the pharmaceutical industry. The same was true for tamoxifen and raloxifene. Now the market may be overcrowded but there have been advances. The ideal SERM is

illustrated in Fig. 7 with the goal to achieve an improvement on raloxifene, the failed breast cancer drug. Raloxifene is seen to be a safe advance over tamoxifen as there is no endometrial cancer incidence and no rat hepatocarcinogenicity with the former. If we only focus on SERMs that have successfully moved to approval for osteoporosis (or hot flashes in the case of bazedoxifene) it is clear that lasofoxifene has solved additional issues by reducing strokes and reducing CHD. Significant progress has been made. The innovation of using CEE with bazedoxifene to protect the uterus (and breast in early menopausal women) may yet prove to be useful as ERT in younger postmenopausal women.

So if SERMs are currently optimal for the foreseeable future what about “no oestrogen” at all. The aromatase inhibitors have been rigorously tested in clinical trials of treatment and there is a recent trial of letrozole versus placebo in high risk women that has shown promise for future consideration⁹¹. However, despite the positive claims about low side effects such as bone loss, joint pain and vaginal dryness (with the attendant sexual issues), it would be hard to believe that the side effects of the many could ever outweigh the benefits of the few. If large populations are to benefit from AI’s, issues of increased risk of CHD will again demand rigorous monitoring⁹². Good quality of life is essential for chemoprevention. This was the basis, some 60 years ago, for the introduction of ERT/HRT to improve quality of life for the many. Unfortunately oestrogen and HRT have a bad reputation for the growth of breast cancer for the few.⁹³ Nevertheless, there has been a recent surprise and again science is poised to propel innovation forward and make progress.

The surprise was counterintuitive in the oestrogen alone trial of the WHI⁹⁴. The finding that the administration of CEE to postmenopausal hysterectomized women in their late 60’s reduced the incidence of breast cancer and this remained reduced for 5 years after stopping CEE¹³ demands explanation. Clues as to the mechanisms of the paradoxical antitumour effects of low dose oestrogen administration to women in their late sixties come from work of the mechanisms of antihormone resistance during long term therapy¹². Two decades of laboratory study of the consequences of long term SERM therapy demonstrated an evolution of the types of resistance culminating in the discovery of a new biology of

oestrogen induced apoptosis⁹⁵. It appears that the five years of adjuvant antihormone therapy for breast cancer accelerates a process of breast cancer cell survival that is similar to what occurs over the 20 years with long term oestrogen deprivation following the menopause. Physiologically oestrogen deprivation after menopause needs decades to change the cell sensitivity from oestrogen being a survival signal in breast cancer to an apoptotic trigger. In contrast it takes less than a decade to achieve the same effect on breast cancer with antihormone therapy. The WHI results and the associated laboratory evidence now pose a provocative dilemma in the era of “individualized” medicine. The application of low dose (physiologic) oestrogen- induced apoptosis has already moved successfully from the laboratory to clinical trial⁹⁶, and is being tested as a “purge strategy” for long term AI adjuvant therapy with 3 month drug holidays annually in the Study of Letrozole Extension (SOLE) trial⁹⁷. Maybe the era of individualized chemoprevention is soon to dawn as we piece together all the advances being made in cancer research and treatment. This era will deploy new knowledge of genetics, lifestyle, detection, and molecular medicine for the right preventative for the right women. If in fact we can understand the mechanism of oestrogen-induced apoptosis⁹⁸ as currently applied to second line treatment after SERMs or aromatase inhibitors and use the knowledge to alternate or “purge” nascent breast cancer cells resistant to SERMs used as long term preventatives with CEE for a few months, this new approach may be added to the armamentarium available to physicians as inexpensive but effective.

A

Acknowledgements: This work (VCJ) was supported by the Department of 1090 Defense Breast Program under Award number BC050277 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen For The Cure Foundation under Award number SAC100009 and the Lombardi Comprehensive Cancer 1095 Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the US Army or the Department of Defense.

1. Lacassagne A. Hormonal Pathogenesis of Adenocarcinoma of the Breast. *Am J Cancer*. 1936;27:217-25.
2. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov*. 2003;2:205-13.
3. Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol*. 2006;147 Suppl 1:S269-76.
4. Adjuvant tamoxifen in the management of operable breast cancer: the Scottish Trial. Report from the Breast Cancer Trials Committee, Scottish Cancer Trials Office (MRC), Edinburgh. *Lancet*. 1987;2:171-5.
5. Fisher B, Costantino J, Redmond C, *et al*. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *N Engl J Med*. 1989;320:479-84.
6. Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev*. 1984;36:245-76.
7. Lerner LJ, Jordan VC. Development of antiestrogens and their use in breast cancer: eighth Cain memorial award lecture. *Cancer Res*. 1990;50:4177-89.
8. Jordan VC. The science of selective estrogen receptor modulators: concept to clinical practice. *Clin Cancer Res*. 2006;12:5010-3.
9. Jordan VC. Chemoprevention of breast cancer with selective oestrogen-receptor modulators. *Nat Rev Cancer*. 2007;7:46-53.
10. Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. *Recent Results Cancer Res*. 1993;127:23-33.
11. Yao K, Lee ES, Bentrem DJ, *et al*. Antitumor action of physiological estradiol on tamoxifen-stimulated breast tumors grown in athymic mice. *Clin Cancer Res*. 2000;6:2028-36.
12. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. *Cancer Prev Res (Phila)*. 2011;4:633-7.
13. LaCroix AZ, Chlebowski RT, Manson JE, *et al*. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy: a randomized controlled trial. *JAMA*. 2011;305:1305-14.
14. Jordan VC. A century of deciphering the control mechanisms of sex steroid action in breast and prostate cancer: the origins of targeted therapy and chemoprevention. *Cancer Res*. 2009;69:1243-54.
15. EBCTG. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet*. 2011;378:771-84.
16. Gottardis MM, Robinson SP, Satyaswaroop PG, *et al*. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res*. 1988;48:812-5.
17. Fornander T, Rutqvist LE, Cedermark B, *et al*. Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. *Lancet*. 1989;1:117-20.
18. Gail MH, Brinton LA, Byar DP, *et al*. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst*. 1989;81:1879-86.
19. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil*. 1967;13:101-19.
20. Consensus conference. Adjuvant chemotherapy for breast cancer. *JAMA*. 1985;254:3461-3.
21. Love RR, Newcomb PA, Wiebe DA, *et al*. Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. *J Natl Cancer Inst*. 1990;82:1327-32.
22. Love RR, Wiebe DA, Newcomb PA, *et al*. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann Intern Med*. 1991;115:860-4.

23. Nordenskjöld B, Rosell J, Rutqvist LE, *et al.* Coronary heart disease mortality after 5 years of adjuvant tamoxifen therapy: Results from a randomized trial. *J Natl Cancer Inst.* 2005;97:1609-10.
24. Hackshaw A, Roughton M, Forsyth S, *et al.* Long-term benefits of 5 years of tamoxifen: 10-year follow-up of a large randomized trial in women at least 50 years of age with early breast cancer. *J Clin Oncol.* 2011;29:1657-63.
25. McDonald CC, Stewart HJ. Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial. The Scottish Breast Cancer Committee. *BMJ.* 1991;303:435-7.
26. Jordan VC, Phelps E, Lindgren JU. Effects of anti-estrogens on bone in castrated and intact female rats. *Breast Cancer Res Treat.* 1987;10:31-5.
27. Turner RT, Wakley GK, Hannon KS, *et al.* Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. *J Bone Miner Res.* 1987;2:449-56.
28. Turner RT, Wakley GK, Hannon KS, *et al.* Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. *Endocrinology.* 1988;122:1146-50.
29. Love RR, Mazess RB, Barden HS, *et al.* Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med.* 1992;326:852-6.
30. Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinomata. *Eur J Cancer.* 1976;12:419-24.
31. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res.* 1987;47:4020-4.
32. Jordan VC, Lababidi MK, Langan-Fahey S. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. *J Natl Cancer Inst.* 1991;83:492-6.
33. Greaves P, Goonetilleke R, Nunn G, *et al.* Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res.* 1993;53:3919-24.
34. Jordan VC. What if tamoxifen (ICI 46,474) had been found to produce rat liver tumors in 1973? A personal perspective. *Ann Oncol.* 1995;6:29-34.
35. Jordan VC, Morrow M. Should clinicians be concerned about the carcinogenic potential of tamoxifen? *Eur J Cancer.* 1994;30A:1714-21.
36. Powles TJ, Hardy JR, Ashley SE, *et al.* A pilot trial to evaluate the acute toxicity and feasibility of tamoxifen for prevention of breast cancer. *Br J Cancer.* 1989;60:126-31.
37. Powles TJ, Ashley S, Tidy A, *et al.* Twenty-year follow-up of the Royal Marsden randomized, double-blinded tamoxifen breast cancer prevention trial. *J Natl Cancer Inst.* 2007;99:283-90.
38. Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst.* 1998;90:1371-88.
39. Fisher B, Costantino JP, Wickerham DL, *et al.* Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst.* 2005;97:1652-62.
40. Cuzick J, Forbes JF, Sestak I, *et al.* Long-term results of tamoxifen prophylaxis for breast cancer--96-month follow-up of the randomized IBIS-I trial. *J Natl Cancer Inst.* 2007;99:272-82.
41. (EBCTCG) EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet.* 2005;365:1687-717.
42. Lewis JS, Jordan VC. Case Histories: Raloxifene. In Taylor J and Triggle, D, eds. *Comprehensive Medicinal Chemistry II*, Volume 8 Elsevier Limited, Oxford, UK 2006:103-21.
43. Black LJ, Jones CD, Falcone JF. Antagonism of estrogen action with a new benzothiophene derived antiestrogen. *Life Sci.* 1983;32:1031-6.
44. Jordan VC. Chemosuppression of breast cancer with tamoxifen: laboratory evidence and future clinical investigations. *Cancer Invest.* 1988;6:589-95.

45. Cummings SR, Eckert S, Krueger KA, *et al.* The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA*. 1999;281:2189-97.
46. Martino S, Cauley JA, Barrett-Connor E, *et al.* Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst*. 2004;96:1751-61.
47. Ettinger B, Black DM, Mitlak BH, *et al.* Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA*. 1999;282:637-45.
48. Barrett-Connor E, Mosca L, Collins P, *et al.* Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med*. 2006;355:125-37.
49. Vogel VG, Costantino JP, Wickerham DL, *et al.* Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA*. 2006;295:2727-41.
50. Vogel VG, Costantino JP, Wickerham DL, *et al.* Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: Preventing breast cancer. *Cancer Prev Res (Phila)*. 2010;3:696-706.
51. Baumler E. *Paul Ehrlich, Scientist for Life*, New York: Holmes & Meier,. 1984:288.
52. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J Med Chem*. 2003;46:1081-111.
53. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *J Med Chem*. 2003;46:883-908.
54. Qu Q, Harkonen PL, Vaananen HK. Comparative effects of estrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. *J Cell Biochem*. 1999;73:500-7.
55. Gennari L, Merlotti D, Valleggi F, *et al.* Ospemifene use in postmenopausal women. *Expert Opin Investig Drugs*. 2009;18:839-49.
56. Taras TL, Wurz GT, DeGregorio MW. In vitro and in vivo biologic effects of Ospemifene (FC-1271a) in breast cancer. *J Steroid Biochem Mol Biol* 2001;77:271-9.
57. Voipio SK, Komi J, Halonen K, *et al.* Effects of ospemifene (FC-1271a) on uterine endometrium, vaginal maturation index, and hormonal status in healthy postmenopausal women. *Maturitas*. 2002;43:207-14.
58. Qu Q, Zheng H, Dahllund J, *et al.* Selective estrogenic effects of a novel triphenylethylene compound, FC1271a, on bone, cholesterol level, and reproductive tissues in intact and ovariectomized rats. *Endocrinology*. 2000;141:809-20.
59. Rutanen EM, Heikkinen J, Halonen K, *et al.* Effects of ospemifene, a novel SERM, on hormones, genital tract, climacteric symptoms, and quality of life in postmenopausal women: a double-blind, randomized trial. . *Menopause*. 2003;10:433-9.
60. Bachmann GA, Komi JO, Group OS. Ospemifene effectively treats vulvovaginal atrophy in postmenopausal women: results from a pivotal phase 3 study. *Menopause*. 2010;17:480-6.
61. Komi J, Lankinen KS, Harkonen P, *et al.* Effects of ospemifene and raloxifene on hormonal status, lipids, genital tract, and tolerability in postmenopausal women. *Menopause*. 2005;12:202-9.
62. Palkowitz AD, Glasebrook AL, Thrasher KJ, *et al.* Discovery and synthesis of [6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxyphenyl)]benzo[b]thiophene: a novel, highly potent, selective estrogen receptor modulator. *J Med Chem*. 1997;40:1407-16.
63. Bryant HU, Glasebrook AL, Yang NN, *et al.* An estrogen receptor basis for raloxifene action in bone. *J Steroid Biochem Mol Biol*. 1999;69:37-44.
64. Grese TA, Cho S, Finley DR, *et al.* Structure-activity relationships of selective estrogen receptor modulators: modifications to the 2-arylbenzothiophene core of raloxifene. *J Med Chem*. 1997;40.

65. Sato M, Turner CH, Wang T, *et al.* LY353381.HCl: a novel raloxifene analog with improved SERM potency and efficacy in vivo. *J Pharmacol Exp Ther.* 1998;287:1-7.
66. Kendler DL, Palacios S, Cox DA, *et al.* Arzoxifene versus raloxifene: effect on bone and safety parameters in postmenopausal women with osteoporosis. *Osteoporos Int.* 2011.
67. Deshmane V, Krishnamurthy S, Melemed AS, *et al.* Phase III Double-Blind Trial of Arzoxifene Compared With Tamoxifen for Locally Advanced or Metastatic Breast Cancer *Journal of Clinical Oncology.* 2007;25:4967-73.
68. Gruber C, Gruber D. Bazedoxifene (Wyeth). *Curr Opin Investig Drugs* 2004;5:1086-93.
69. Miller CP, Collini MD, Tran BD, *et al.* Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem.* 2001;44:1654-7.
70. Komm BS, Kharode YP, Bodine PV, *et al.* Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology* 2005;146:3999-4008.
71. Black LJ, Sato M, Rowley ER, *et al.* Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. *J Clin Invest* 1994;93:63-9.
72. Ke HZ, Paralkar VM, Grasser WA, *et al.* Effects of CP-336,156, a new, nonsteroidal estrogen agonist/antagonist, on bone, serum cholesterol, uterus and body composition in rat models. *Endocrinology.* 1998;139.
73. Kharode Y, Bodine PV, Miller CP, *et al.* The pairing of a selective estrogen receptor modulator, bazedoxifene, with conjugated estrogens as a new paradigm for the treatment of menopausal symptoms and osteoporosis prevention. *Endocrinology.* 2008;149:6084-91.
74. Komm B, Kharode Y, Bodine P, *et al.* Combining a SERM with conjugated estrogens (CE) to improve the SERM profile: not all SERMs may succeed. *J Bone Miner Res.* 2003;25.
75. Miller PD, Chines AA, Christiansen C, *et al.* Effects of bazedoxifene on BMD and bone turnover in postmenopausal women: 2-yr results of a randomized, double-blind, placebo-, and active-controlled study. *J Bone Miner Res.* 2008;23:525-35.
76. Pinkerton JV, Archer DF, Utian WH, *et al.* Bazedoxifene effects on the reproductive tract in postmenopausal women at risk for osteoporosis. *Menopause.* 2009;16:1102-8.
77. Silverman SL, Christiansen C, Genant HK, *et al.* Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. *J Bone Miner Res.* 2008;23:1923-34.
78. de Villiers TJ, Chines AA, Palacios S, *et al.* Safety and tolerability of bazedoxifene in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled phase 3 trial. *Osteoporos Int.* 2011;22:567-76.
79. Grady D, Ettinger B, Moscarelli E, *et al.* Safety and adverse effects associated with raloxifene: multiple outcomes of raloxifene evaluation. *Obstet Gynecol* 2004;104:837-44.
80. Cummings SR, Ensrud K, Delmas PD, *et al.* Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med.* 2010;362:686-96.
81. http://www.ukmi.nhs.uk/applications/ndo/record_view_open.asp?newDrugID=3184 Af. UK Medicines Information New Drugs Online. New Drugs Online Report for lasofoxifene. [cited 2011 29 September].
82. Maeda T, Ke HZ, Simmons H, *et al.* Lasofoxifene, a next generation estrogen receptor modulator: preclinical studies. 2004;14:85-93. *Clin Calcium.* 2004;14:85-93.
83. Moffett A, Ettinger M, Bolognese M, *et al.* Lasofoxifene, a next generation SERM, is effective in preventing loss of BMD and reducing LDL-C in postmenopausal women. *J Bone Min Res.* 2004;19:S96.
84. Lee A, Radecki D, Wolter K. Lasofoxifene Phase II and Phase III clinical trial design and strategy. *J Bone Min Res.* 2005;20:M384.

85. Eastell R, Reid DM, Vukicevic S, *et al.* The effects of lasofoxifene on bone turnover markers: the PEARL trial. *J Bone Min Res.* 2008;23.
86. Cummings SR, Ensrud K, Delmas PD, *et al.* Lasofoxifene in postmenopausal women with osteoporosis. *N Engl J Med.* 2010;362:686-96.
87. LaCroix AZ, Powles T, Osborne CK, *et al.* Breast cancer incidence in the randomized PEARL trial of lasofoxifene in postmenopausal osteoporotic women. *J Natl Cancer Inst.* 2010;102:1706-15.
88. McClung M SE, Cummings S, *et al.* Lasofoxifene increased BMD of the spine and hip and decreased bone turnover markers in postmenopausal women with low or normal BMD. *J Bone Min Res.* 2005;20:S97.
89. Davidson M, Moffett A, Welty F, *et al.* Extraskelatal effects of lasofoxifene on postmenopausal women. *J Bone Min Res.* 2005;20:S173.
90. McClung MR, Siris E, Cummings S, *et al.* Prevention of bone loss in postmenopausal women treated with lasofoxifene compared with raloxifene. *Menopause.* 2006;13:377-86.
91. Goss PE, Ingle JN, Ales-Martinez JE, *et al.* Exemestane for breast-cancer prevention in postmenopausal women. *N Engl J Med.* 2011;364:2381-91.
92. Cuppone F, Bria E, Verma S, *et al.* Do adjuvant aromatase inhibitors increase the cardiovascular risk in postmenopausal women with early breast cancer? Meta-analysis of randomized trials. *Cancer.* 2008;112:260-7.
93. Chlebowski RT, Kuller LH, Prentice RL, *et al.* Breast cancer after use of estrogen plus progestin in postmenopausal women. *N Engl J Med.* 2009;360:573-87.
94. The women's health initiative steering committee. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: The Women's Health Initiative Randomized Control Trial. *JAMA.* 2004;291:1701-12.
95. Jordan VC. Selective estrogen receptor modulation: concept and consequences in cancer. *Cancer Cell.* 2004;5:207-13.
96. Ellis MJ, Gao F, Dehdashti F, *et al.* Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA.* 2009;302:774-80.
97. Jordan VC, Ford LG. Paradoxical clinical effect of estrogen on breast cancer risk: a "new" biology of estrogen-induced apoptosis. *Cancer Prev Res (Phila).* 2011;4:633-7.
98. Ariazi EA, Brailoiu E, Yerrum S, *et al.* The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res.* 2010;70:1184-94.

Figure Legends

Figure 1.

A comparison of the structures of the potent steroidal oestrogen 17 β -oestradiol and the non-steroidal antioestrogens (now called SERMs) tamoxifen and raloxifene. Tamoxifen and raloxifene are both approved in the U.S. for the reduction of risk for breast cancer in high risk pre and postmenopausal women or postmenopausal women alone respectively.

Figure 2.

The risk requirements for recruitment to the National Surgical Breast and Bowel Project (NSABP)/National Cancer Institute (NCI) study P-1 to determine the worth of tamoxifen for preventing breast cancer in high risk pre and postmenopausal women ³⁸.

Figure 3.

The total and age related incidence of endometrial cancer in the NSABP/NCI P-1 chemoprevention trial ³⁹. Premenopausal women have no increased risk of developing endometrial cancer during or following 5 years of tamoxifen treatment.

Figure 4.

The annual accumulative incidence breast cancers represented as a percent of affected randomized patients in the Multiple Outcomes of Raloxifene Evaluation (MORE) that randomized women with an increased risk for osteoporotic fractures to placebo (2576 women) or raloxifene (5129 women) ⁴⁵.

Figure 5.

The cumulative incidence of invasive breast cancer for the combined MORE and Continuing Outcomes Relevant to Evista (CORE) studies. Shown are patients at high risk for osteoporotic fractures receiving either placebo or raloxifene (60mg daily) ⁴⁶.

Figure 6.

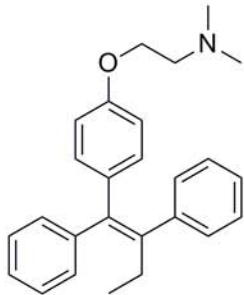
The design of the Study of Tamoxifen and Raloxifene (STAR). The STAR trial for postmenopausal women at elevated risk for breast cancer had fewer serious side effects when taking raloxifene but a similar reduction in the incidence of breast cancer⁴⁹. However, after stopping the SERM treatment, the antitumour action of raloxifene was not maintained⁵⁰ so continuous treatment with raloxifene is recommended (as this is the approval for the treatment and prevention of osteoporosis).

Figure 7.

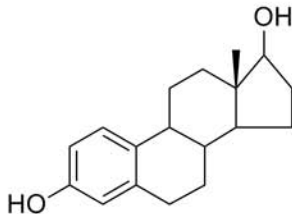
A comparison of the good and bad aspects of hormone replacement therapy (HRT) and current Selective Oestrogen Receptor Modulators (SERMs) tested in postmenopausal women. On the right is the ideal SERM of the future reproduced with permission from⁹⁵.

Figure 8.

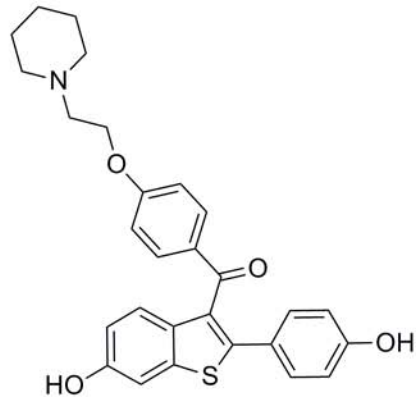
The structures of SERMs that have completed clinical testing over the last decade. Arzoxifene has not been pursued for clinical use and ospemifene is targeted for an application for vaginal atrophy. Lasofoxifene is the newest SERM thus far to attain the pharmacologic profile of an ideal SERM (Fig. 7). Bazedoxifene is targeted for either a treatment and prevention for osteoporosis or with conjugated equine oestrogen as an oestrogen replacement therapy for hot flashes.



Tamoxifen
Originally ICI 46,474
a failed "morning after" pill



17 β -Estradiol



Raloxifene
originally Keoxifene
a failed breast cancer drug

Potential Participants

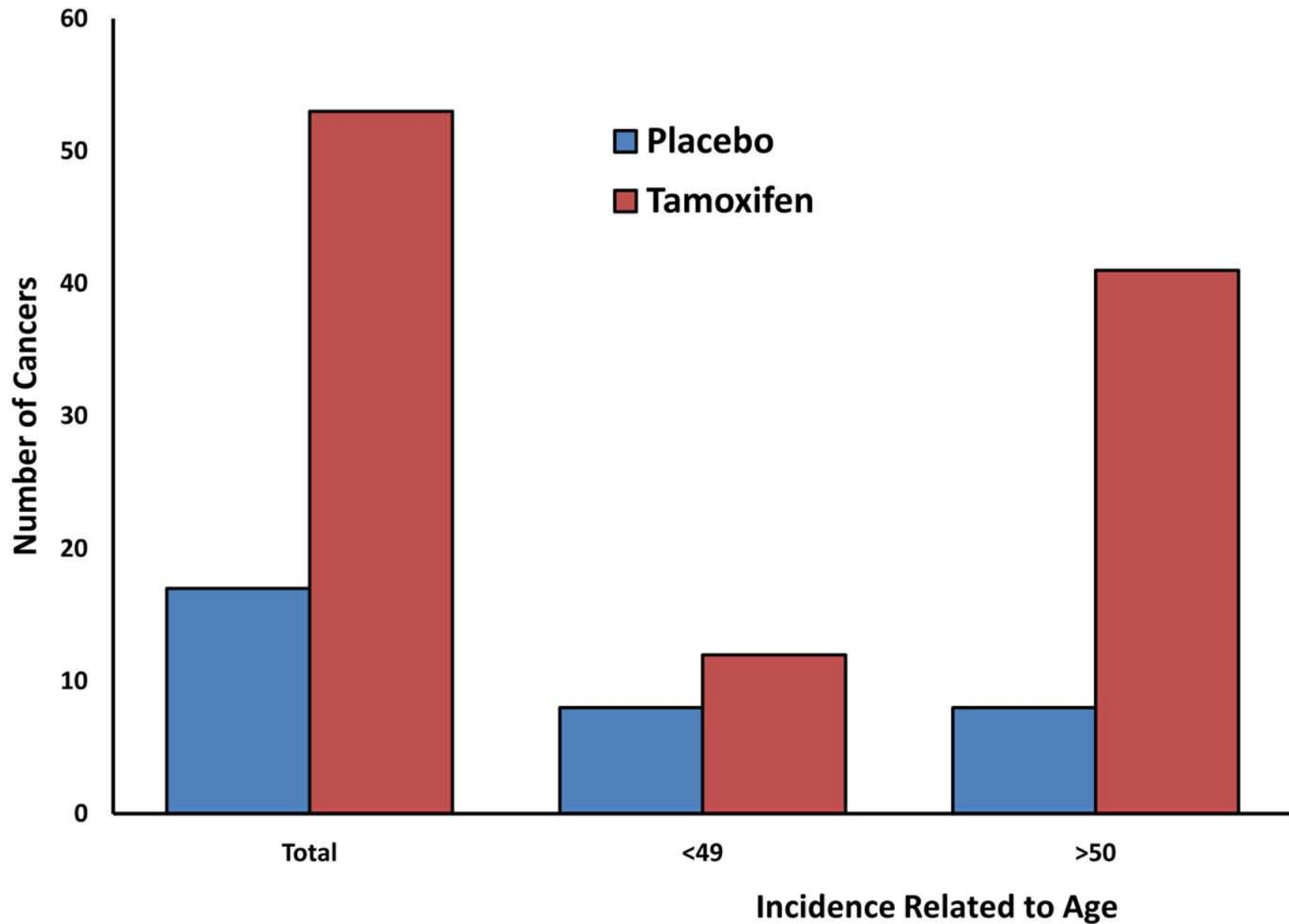
>60 years old - with/without risk factors
35-59 years old - with risk factors

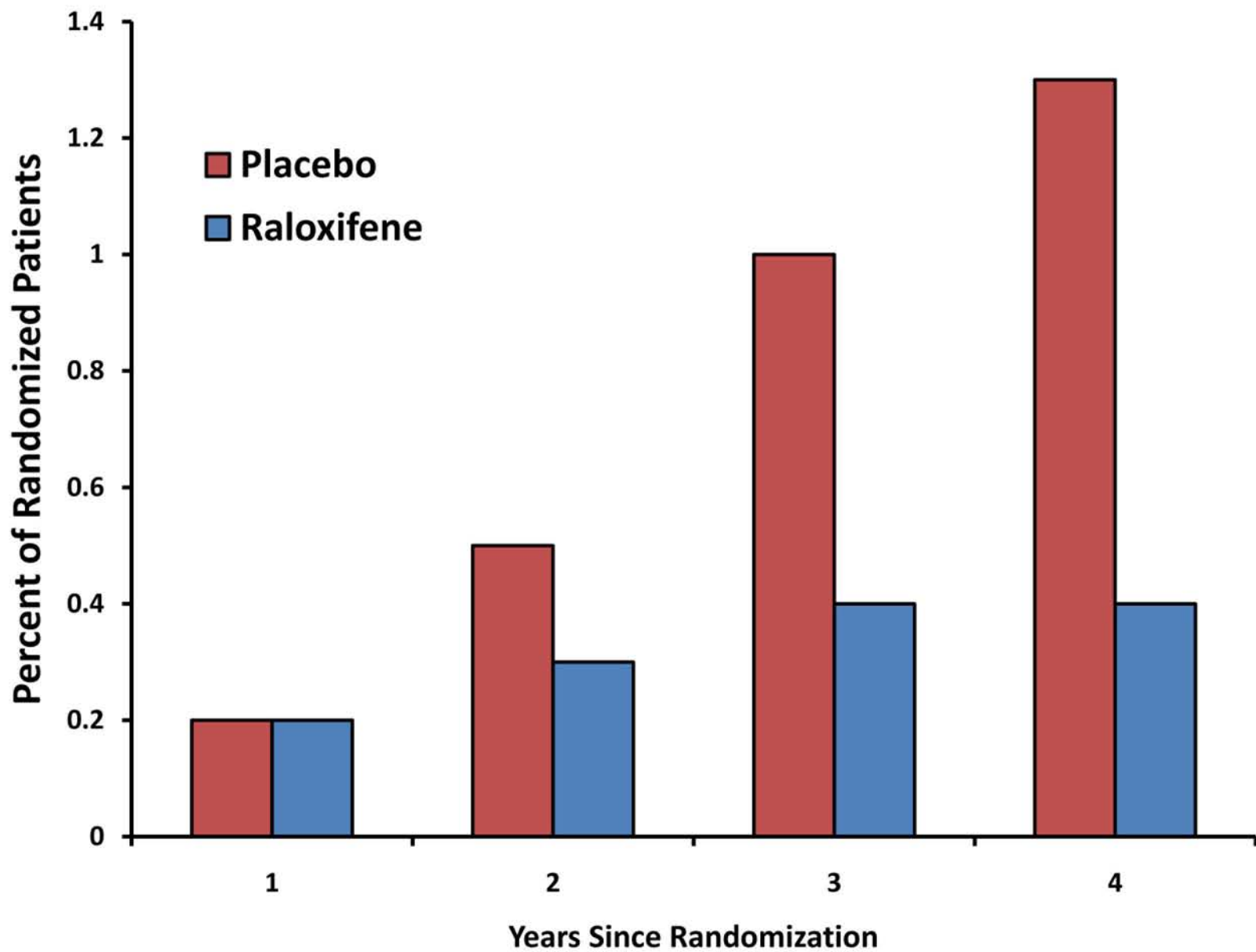
- LCIS
- relative with breast cancer
- Breast Biopsy
- Atypical hyperplasia
- > 25 years 1st child
- No children
- Menarche before age 12

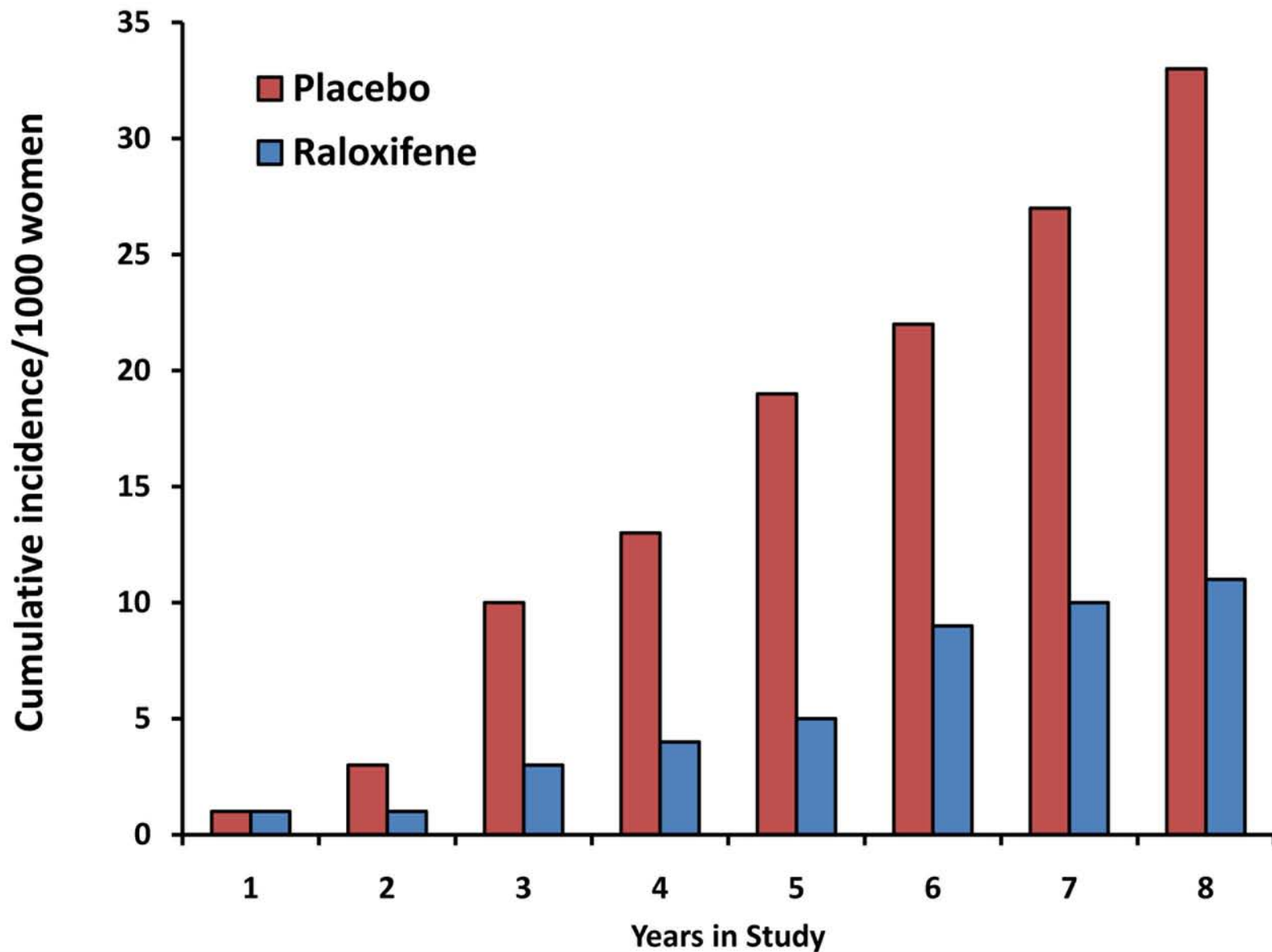
RANDOMIZE
13,800 women

Placebo

Tamoxifen
20 mg/daily
5 years







**Postmenopausal
High Risk Women
19,000**

**Tamoxifen
(20 mg daily)**

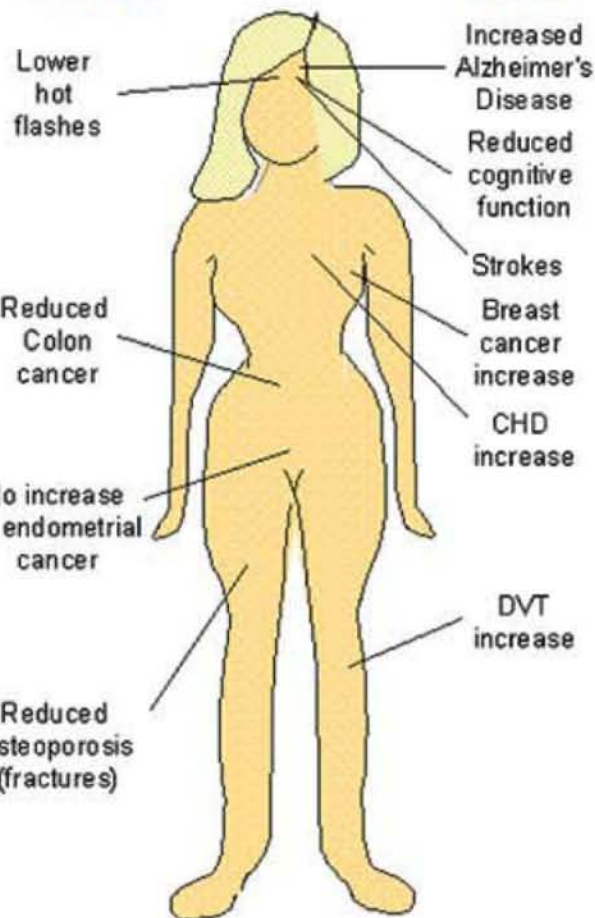
Five Years Treatment: Two Years Of Follow Up

**Raloxifene
(60 mg daily)**

**Endometrial Surveillance: Self Reporting
Subproject To Compare Endometrial
And Uterine Characteristics**

HRT

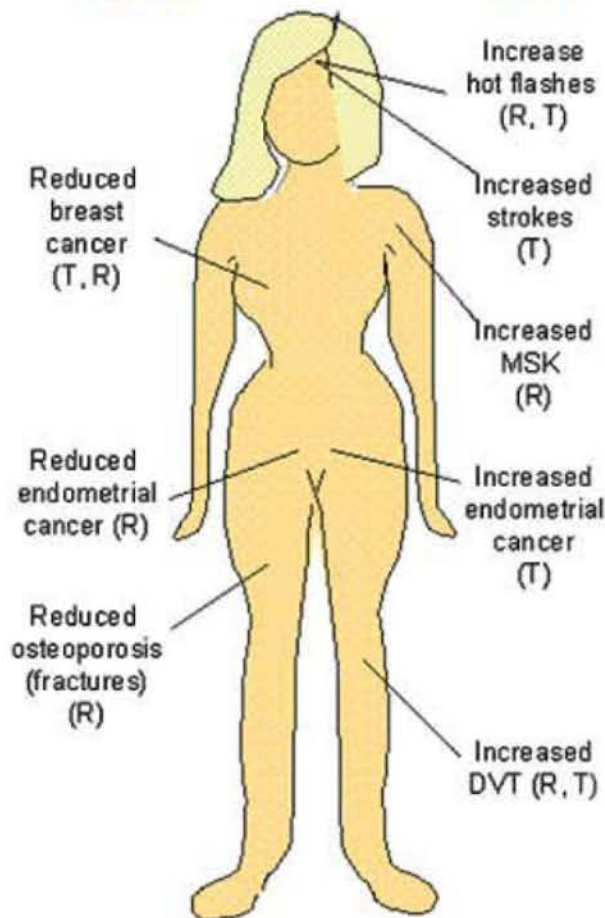
Good



Bad

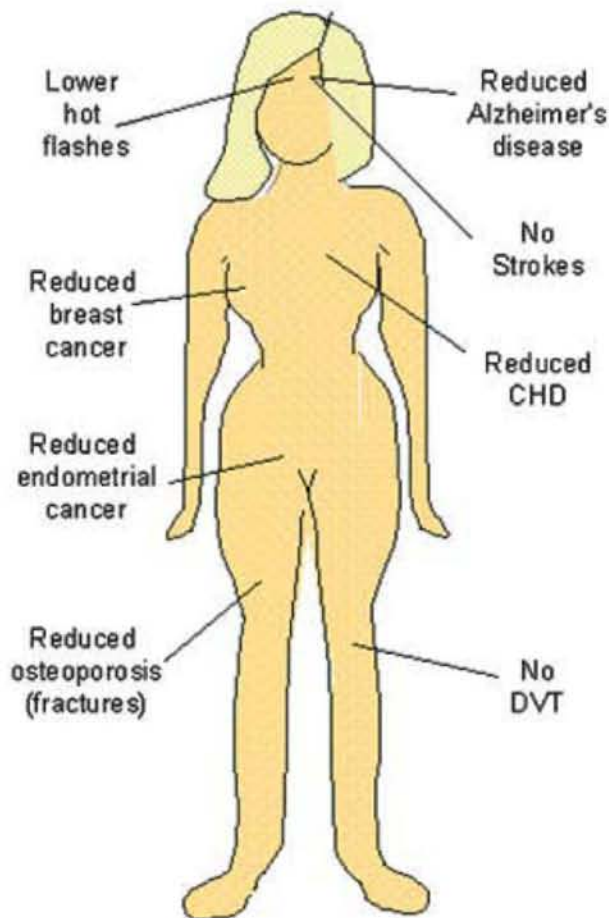
SERM (T Tamoxifen) (R Raloxifene)

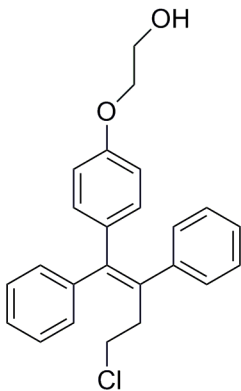
Good



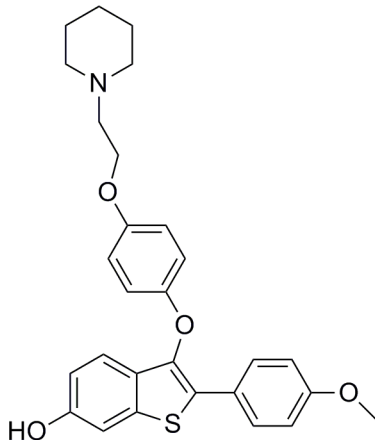
Bad

Ideal SERM

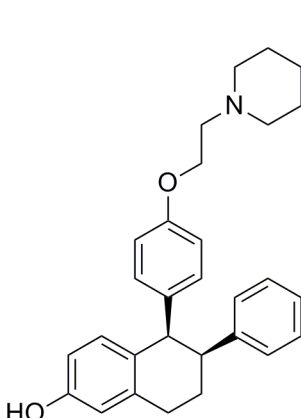




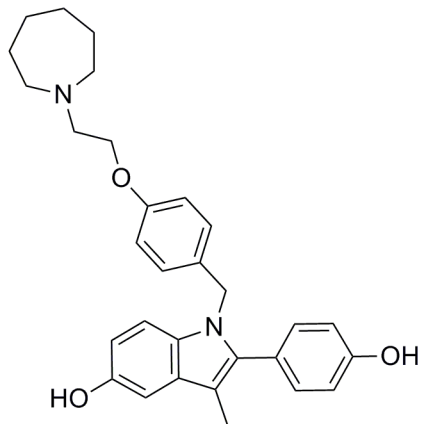
Ospemifene



Arzoxifene




Lasofoxifene



Bazedoxifene

AUTHOR QUERY FORM

	Journal: EJC Article Number: 8337	Please e-mail or fax your responses and any corrections to: E-mail: corrections.esco@elsevier.sps.co.in Fax: +31 2048 52799
---	--	---

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. Note: if you opt to annotate the file with software other than Adobe Reader then please also highlight the appropriate place in the PDF file. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult <http://www.elsevier.com/artworkinstructions>.

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	Please define the abbreviation PP2.
Q2	Please define the abbreviation PR.
Q3	Please define the abbreviation SDS.
Q4	Please define the abbreviation DMSO
Q5	Would you consider changing the phrase 'after normalised_' to 'after being normalised' in the sentence Althoughcell lines....._ ? Please check, and correct if necessary.
Q6	Please check the edit(s) made in the sentence Therefore.....effects., and correct if necessary.
Q7	Does SFS stands for 'dextran-coated charcoal-stripped foetal bovine serum'? Please clarify.
	<div data-bbox="400 1900 965 2002"> <p>Please check this box if you have no corrections to make to the PDF file</p> <input data-bbox="852 1917 924 1981" type="checkbox"/> </div>

Thank you for your assistance.



Available at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.ejconline.com



Modulating therapeutic effects of the c-Src inhibitor via oestrogen receptor and human epidermal growth factor receptor 2 in breast cancer cell lines

Ping Fan, Russell E. McDaniel, Helen R. Kim, Dionyssia Clagett, Bassem Haddad, V. Craig Jordan*

Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20057, USA

KEYWORDS

c-Src
Oestrogen receptor
HER2
Breast cancer cell lines

Abstract Purpose: c-Src is an important adapter protein with oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2), which validates it as an attractive target for the treatment of breast cancer. A specific c-Src inhibitor, PP2, was utilised to block c-Src activity to identify targeted vulnerabilities affected by ER and HER2 in a panel of breast cancer cell lines.

Methods: ER, growth factor receptors and signalling pathways were detected by Western-blot. The DNA content of the cells was determined by using a DNA fluorescence quantitation kit. Cell cycles were analysed by flow cytometry.

Results: The antiproliferative effect of PP2 closely correlated with the inhibition of c-Src mediated extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and/or phosphoinositide 3-kinase (PI3K)/Akt growth pathways. Inhibition of c-Src tyrosine kinase predominantly blocked ER negative breast cancer cell growth, particularly the triple (i.e. ER, PR, and HER2) negative cells. In contrast, ER negative Sk-Br-3 cells with highest HER2 phosphorylation were resistant to PP2, in which hyper-activated HER2 directly regulated growth pathways. However, blocking c-Src recovered ER expression and down-regulated HER2 which made Sk-Br-3 cells regain responsiveness to 4-hydroxytamoxifen. The majority of ER positive cells were not sensitive to PP2 regardless of wild-type or endocrine resistant cell lines.

Conclusions: c-Src mediates the essential role of growth pathways in ER negative breast cancer cells. The ER positive and HER2 over-activation are two important predictive biomarkers for the resistance to a c-Src inhibitor. These data provided an important therapeutic rationale for patient selection in clinical trials with c-Src inhibitors in breast cancer.

© 2012 Published by Elsevier Ltd.

* Corresponding author: Address: Lombardi Comprehensive Cancer Center, Georgetown University, E507A Research Bldg, 3970 Reservoir RD NW, Washington, DC 20057, USA. Tel.: +1 202 687 2897.

E-mail address: vcj2@georgetown.edu (V. Craig Jordan).

1. Introduction

Targeting oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) are two successful therapies in the treatment of breast cancer patients expressing relevant target molecules.^{1,2} c-Src is a ubiquitously expressed intracellular tyrosine kinase that regulates protein–protein interactions and participates as a convergence point in different signalling pathways.³ c-Src functions as an important adapter protein between ER and receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and HER2 in breast cancer.^{4–6} In this regard, c-Src acts as a critical component of the signalling cascades initiated by ER and HER2 to activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways,^{6,7} both of which cause ER phosphorylation and ER-dependent gene transcription.⁷

Observations *in vitro* also support that multiple levels of association exist among ER, HER2 and c-Src in breast cancer. Targeting ER with tamoxifen increases c-Src activity which enhances cellular invasion and motility in breast cancer cells.^{8,9} Furthermore, c-Src is shown to be critical in mediating tamoxifen resistance since blocking its activity reverses tamoxifen resistance.¹⁰ A recent report indicates that c-Src is a common node downstream of multiple trastuzumab (targeting HER2) resistance pathways.¹¹ These observations highlight c-Src as an important therapeutic target for the treatment of human breast cancer.

Dasatinib, a potent oral inhibitor of c-Src family tyrosine kinase, is approved for clinical use in imatinib-resistant and -intolerant chronic myeloid leukaemia and solid tumour.^{12,13} Preclinical studies in breast cancer cell lines have shown that basal like triple negative (i.e. ER, PR and HER2) breast cancer may have preferential sensitivity to the c-Src inhibitor.^{14,15} Two parallel phase II monotherapy studies of dasatinib in breast cancer were initiated in different breast cancer subtypes. In patients with triple-negative breast cancer (TNBC), dasatinib has good tolerability and modest activity,¹⁶ whereas dasatinib has limited single-agent activity in patients with HER2 positive and/or hormone receptors (HR) positive advanced breast cancer.¹⁷ These findings imply that HR and HER2 may prevent the therapeutic effects of the c-Src inhibitor in breast cancer. Thus, there is a need to identify patients who are unlikely to respond to the c-Src inhibitor treatment. More importantly, factors that cause c-Src inhibitor resistance will serve as molecular targets to improve the action of c-Src inhibitors. Unfortunately, there is little understanding of resistance to the c-Src inhibitors in breast cancer cells. Chen et al.¹⁸ have demonstrated that acquired resistance to AZD 0530 (a c-Src inhibitor) can be mediated through activation of mitogen-activated protein kinase kinase (MEK) and PI3K pathways thus these may prove to

be future therapeutic targets to improve the c-Src inhibitor sensitivity.

The goal of this study is to identify biological markers of resistance to a c-Src inhibitor in a panel of wild-type and long-term oestrogen deprived breast cancer cell lines. We demonstrate that c-Src has an essential role in mediating the growth pathways of ER negative breast cancer cells. ER positive and HER2 over-activation reduce the responsiveness to the c-Src inhibitor. Indeed, c-Src controls oestrogen action in ER positive long-term oestrogen deprived resistant cells. Our data provide an important therapeutic rationale for patient selection in future clinical trials of c-Src inhibitors in breast cancer.

2. Materials and methods

2.1. Materials

The c-Src inhibitor PP2 was purchased from CalBiochem (San Diego, CA). Sources of antibodies for Western blot are as follows: ER α (sc-544) and PR (sc-810) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Total MAPK antibody (#9102), phosphorylation MAPK (#9101), total Akt (#9272), phosphorylated AktSer473 (#9271), phosphorylated c-SrcTyr416 (#2101L) antibodies and secondary antibodies conjugated with horseradish peroxidase (rabbit #7074, mouse #7076) were from Cell Signalling Technology (Beverly, MA). Phosphorylated HER2 Tyr1248 and total c-Src mouse (GD11) antibodies were from Millipore (Temecula, CA). Antibodies to HER2 (Ab18) and EGFR (Ab15) were from NeoMarkers (Fremont, CA).

2.2. Cells and culture conditions

Briefly, MCF-7:WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by E₂,¹⁹ were used in all experiments indicating MCF-7 and T47D cells. ZR-75-1, BT474 and Sk-Br-3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231(10A) cells,²⁰ clonally selected from parental MDA-MB-231 cells (obtained from ATCC), were used in this study indicating MDA-MB-231 cells. MCF-7:5C and MCF-7:2A cells were cloned from E₂ deprived MCF-7 cells and maintained in E₂-free Roswell Park Memorial Institute (RPMI) medium which is phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped foetal bovine serum (SFS).^{21,22} T47D:C42 cells were cloned from E₂ deprived T47D cells and maintained in E₂-free RPMI 1640 medium.²³ Pure antioestrogen fulvestrant resistant cell line MCF-7/F was derived from MCF-7 which was maintained in phenol red RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS).²⁴

2.3. Cell proliferation assays

Cell DNA content was determined as a measure of cell proliferation using the Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA)²⁵

2.4. Immunoblotting

Proteins were extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set I and Set II (Calbiochem, San Diego, CA). Total protein content of the lysate was determined by a standard BCA assay using the reagent from Bio-Rad Laboratories (Hercules, CA). Fifty micrograms of total protein was separated on 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies followed by incubation with secondary antibody conjugated with HRP and reaction with Western LightingTM plus-ECL enhanced chemiluminescent substrate (PerkinElmer Inc., Waltham MA). Protein bands were visualised by exposing the membrane to X-ray film.

2.5. Cell cycles analysis

Briefly, Sk-Br-3, BT474, T47D:C42 and MDA-MB-231 cells were cultured in dishes. They were treated with vehicle (0.1% DMSO), lapatinib (1 μ M) and PP2 (5 μ M) for 24 h respectively. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with propidium iodide (PI), cells were analysed using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson, San Jose, CA), and the data were analysed with CellQuest software.

2.6. Quantitative real-time RT-PCR

Cells were harvested in TRIzol. Total RNA, isolated with an RNeasy Micro kit (Qiagen, Valencia, CA), was converted to first-strand cDNA using a kit from Applied Biosystem (Foster City, CA). Quantitative real-time polymerase chain reaction (RT-PCR) assays were done with the SYBR Green PCR Master Mixes (Applied Biosystems, Foster City, CA) and a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The PUM1 forward primer was 5'-AATGCAGGCGC-GAGAAAT-3', PUM1 reverse primer was 5'-TTGTGCAGCTGAGGAATAATGA-3'. The ER α forward primer was 5'-GGAGGGCAGGGGTGAA-3', ER α reverse primer was 5'-GGCCAGGCTGTTCTTCTTAGA-3'. All the data were normalised by PUM1.

2.7. Statistical Analysis

All reported values are the means \pm SE. Statistical comparisons were determined with two-tailed Student's

t tests. Results were considered statistically significant if the *P* value was <0.05 .

3. Results

3.1. Baseline levels of ER, HER2 and c-Src activation in a panel of breast cancer cell lines

We addressed the question whether expression of ER and growth factor receptors would affect the therapeutic effects of the c-Src inhibitors in breast cancer cells. To answer this question, a panel of wild-type (MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231 and Sk-Br-3), long-term oestrogen deprived (MCF-7:5C, MCF-7:2A and T47D:C42) and pure antioestrogen ICI 182,780 resistant (MCF-7/F) breast cancer cell lines were investigated. Baseline levels of ER, HER2, EGFR and c-Src were measured by immunoblot analysis. They all keep their biological characteristics with differential levels of ER, PR, HER2 and EGFR (Supplementary Fig. S1A and S1B). All cell lines expressed detectable levels of total c-Src, whereas they manifested different levels of phosphorylated c-Src (Supplementary Fig. S1C). Although there is no clear relationship between c-Src phosphorylation and HR expression (Supplementary Fig. S1D) after normalised by total c-Src among tested cell lines, interestingly, we observe that c-Src is activated in resistant cell lines compared with respective parental cell lines (MCF-7:5C, MCF-7:2A and MCF-7/F versus MCF-7, T47D:C42 versus T47D). The DNA fingerprinting pattern of all cell lines is consistent with the report by the ATCC (Supplementary Fig. S2).

3.2. Inhibitory effects of the c-Src inhibitor on ER positive wild-type breast cancer cells

All ER positive wild-type breast cancer cells were cultured in oestrogenised medium. The specific c-Src inhibitor, PP2, effectively blocked phosphorylation of c-Src in all cell lines (Fig. 1A). However, PP2 could not inhibit all cell growth (Fig. 1B). T47D and BT474 cells were responsive to PP2 with 50% and 40% inhibition after 7 days treatment, respectively (Fig. 1B), whereas MCF-7 and ZR-75-1 cells were resistant to PP2 treatment (Fig. 1B). Further investigation showed that antiproliferative effects of PP2 were correlated with inhibition of extracellular signal-regulated kinase/mitogen-activated protein kinase ERK/MAPK and/or phosphoinositide 3-kinase (PI3K)/Akt pathways. PP2 could not continuously block growth pathways in resistant cells such as MCF-7 and ZR-75-1 (Fig. 1C). In contrast, PP2 effectively inhibited both signalling pathways in T47D and BT474 cells (Fig. 1C).

3.3. Inhibitory effects of the c-Src inhibitor varied under conditions with or without basal E_2 in ER positive wild-type breast cancer cells

Since basal oestrogen levels in the culture medium affect the biological function of the ER positive wild-type breast cancer cells¹⁹ (Supplementary Fig. S3), we investigated inhibitory effects of the c-Src inhibitor on ER positive wild-type cells under conditions with (10% FBS) or without (10% SFS) basal oestrogen. Two distinct modulations of c-Src phosphorylation existed in ER positive wild-type cells after short-term absence of E_2 . MCF-7 and ZR-75-1 cells had the same pattern with enhanced c-Src phosphorylation, conversely, c-Src phosphorylation was down-regulated in T47D and BT474 cells (Fig. 2A). The

PP2 effectively blocked c-Src phosphorylation in four wild-type breast cancer cells under conditions with 10% SFS (Fig. 2B). However, inhibition by PP2 varied in ER positive wild-type cells under these two conditions (Fig. 2C). MCF-7 cells were effectively responsive to PP2 under conditions without basal E_2 (10% SFS), conversely, T47D cells were completely resistant to PP2 in phenol red free medium (Fig. 2C). Four ER positive wild-type breast cancer cells were stimulated by E_2 to grow with different sensitivity (Fig. 2D). Notably, PP2 could not block the proliferation induced by E_2 in MCF-7 and ZR-75-1 cells but partially abolished E_2 stimulation in T47D and BT474 cells (Fig. 2D). These results indicated that c-Src might play a distinct role in mediating E_2 signalling in wild-type cells.^{4,26}

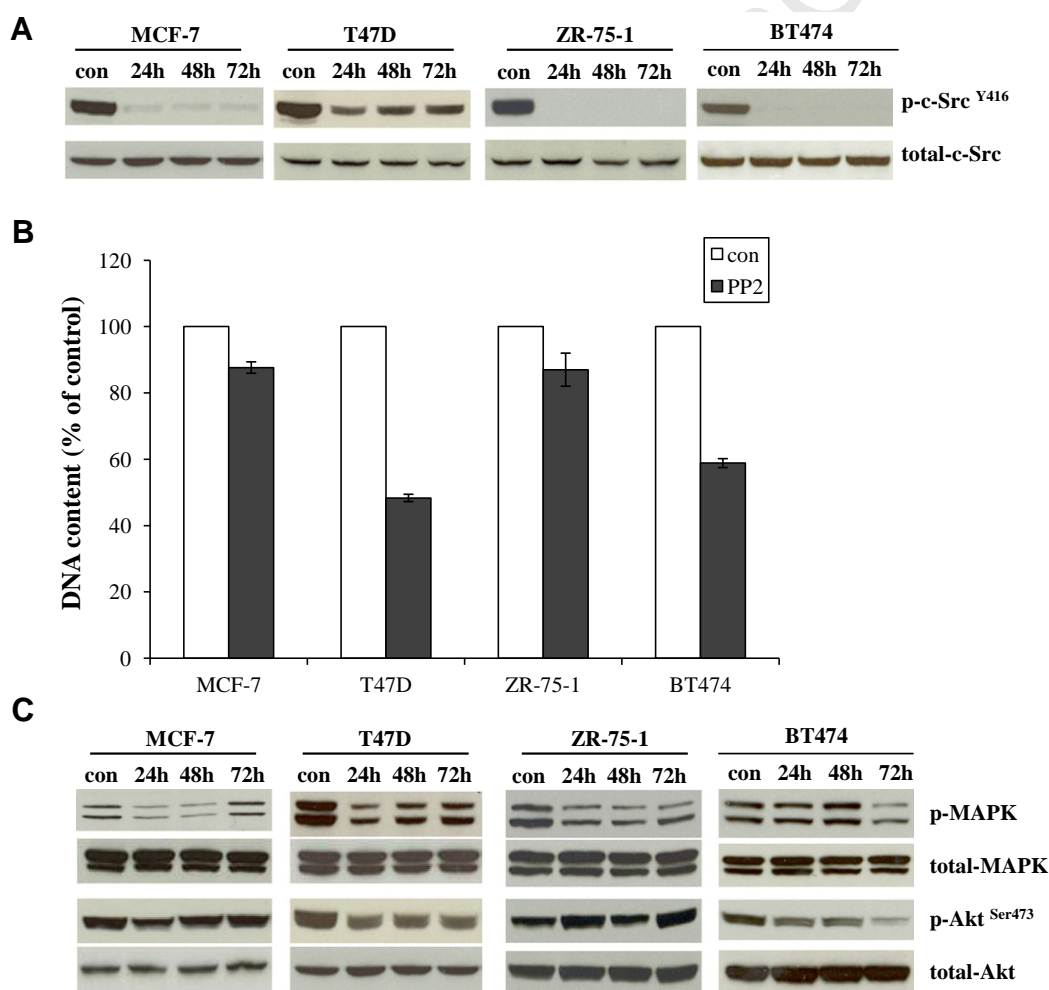


Fig. 1. Effects of the c-Src inhibitor on oestrogen receptor (ER) positive wild-type cell lines. (A) Blocking c-Src phosphorylation in ER positive wild-type cell lines by PP2. ER positive wild-type cells were treated with PP2 (5 μ M) in oestrogenised medium at time points as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (B) Inhibitory effects of PP2 on wild-type ER positive cells. Wild-type ER positive cells were seeded in 24-well plates in triplicate in oestrogenised medium. After 1 day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μ M) respectively. The cells were harvested after 7 days treatment and total DNA was determined using a DNA fluorescence quantitation kit. (C) Signalling pathways change in ER positive wild-type cells after PP2 treatment. Cell lysates were harvested as above. Phosphorylated mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt was used for loading controls.

3.4. Effects of the c-Src inhibitor on ER positive long-term oestrogen deprived breast cancer cells

In two long-term oestrogen deprived breast cancer cells (MCF-7:5C and MCF-7:2A), that overexpress ER, PP2 could block c-Src activation (Fig. 3A) and abolished about 25% of proliferation in MCF-7:5C cells but without any inhibition in MCF-7:2A cells (Fig. 3B). The inhibitory effects of PP2 were consistent with blocking growth pathways in different cells. Phosphorylated Akt was abolished in MCF-7:5C cells but without continuous inhibition of MAPK. PP2 could not continuously block both growth pathways in MCF-7:2A cells (Fig. 3C). Our previous data showed that E₂ has therapeutic function to induce apoptosis in long-term E₂ deprived breast cancer cells.²⁵ We reasoned that a combination of PP2 with E₂ would enhance E₂-induced apoptosis. Surprisingly, PP2 did not enhance the growth inhibitory effects of E₂ on these two cell lines but

blocked the growth inhibition induced by E₂ (Fig. 3D). These data implied that E₂-triggered apoptosis might be utilising c-Src tyrosine kinase as an important signalling pathway. We are currently investigating the mechanisms of how the c-Src inhibitor blocks E₂-triggered apoptosis.

3.5. The c-Src inhibitor effectively blocked ER negative breast cancer cell growth

The inhibitory effects of the c-Src inhibitor, PP2, on ER negative breast cancer cell lines were examined in two wild-type MDA-MB-231 and Sk-Br-3 and two resistant cell lines MCF-7/F (ICI 182,780 resistance) and T47D:C42 (long-term oestrogen deprived). PP2 blocked the phosphorylation of c-Src in all ER negative cells (Fig. 4A). However, the growth inhibitory effects of the c-Src inhibitor were different. PP2 could inhibit 80% of cell growth in MDA-MB-231 cells. In contrast,

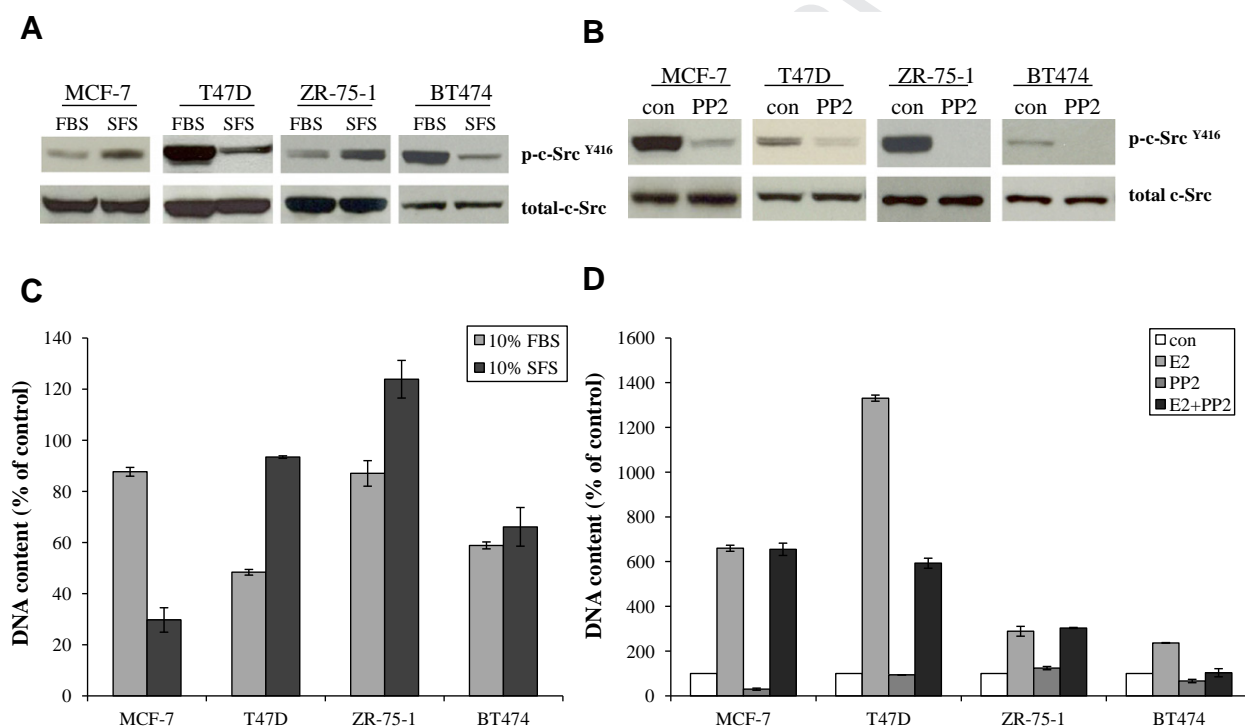


Fig. 2. Effects of the c-Src inhibitor on oestrogen receptor (ER) positive wild-type cell lines under conditions with or without basal E₂. (A) c-Src phosphorylation changed after short-term absence of E₂ in ER positive wild-type cells. Wild-type ER positive cells were cultured under conditions with basal oestrogen (10% foetal bovine serum (FBS)) or without basal oestrogen (10% dextran-coated charcoal-stripped foetal bovine serum (SFS)) for 3 days, respectively. Cell lysates were harvested. Phosphorylated c-Src was examined by immunoblotting with primary antibody. Immunoblotting for total c-Src was determined as loading control. (B) Blocking c-Src phosphorylation in ER positive wild-type cell lines by PP2 under the conditions without basal oestrogen. Wild-type ER positive cells were cultured under the conditions without basal oestrogen (10% SFS) for 3 days. Then cells were treated with PP2 (5 μ M) in 10% SFS medium for 24 h and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (C) Growth inhibitory effects of PP2 on ER positive wild-type cells under conditions with or without basal E₂. Wild-type ER positive cells were cultured under conditions with basal oestrogen (10% FBS) or without basal oestrogen (10% SFS) for 3 days, respectively. Then, they were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μ M) in oestrogenised medium (10% FBS) or E₂ free medium (10% SFS), respectively. The cells were harvested after 7 days treatment and total DNA was determined as above. (D) The PP2 had different effects on E₂ stimulation in ER positive wild-type cells. Wild-type ER positive cells were changed to E₂ free medium for 3 days. Then, they were seeded in 24-well plates. After one day, the cells were treated with vehicle (0.1% EtOH), E₂ (10⁻⁹ mol/L), PP2 (5 μ M) and E₂ (10⁻⁹ mol/L) plus PP2 (5 μ M) respectively in E₂ free culture medium. The cells were harvested after 7 days treatment and total DNA was determined as above.

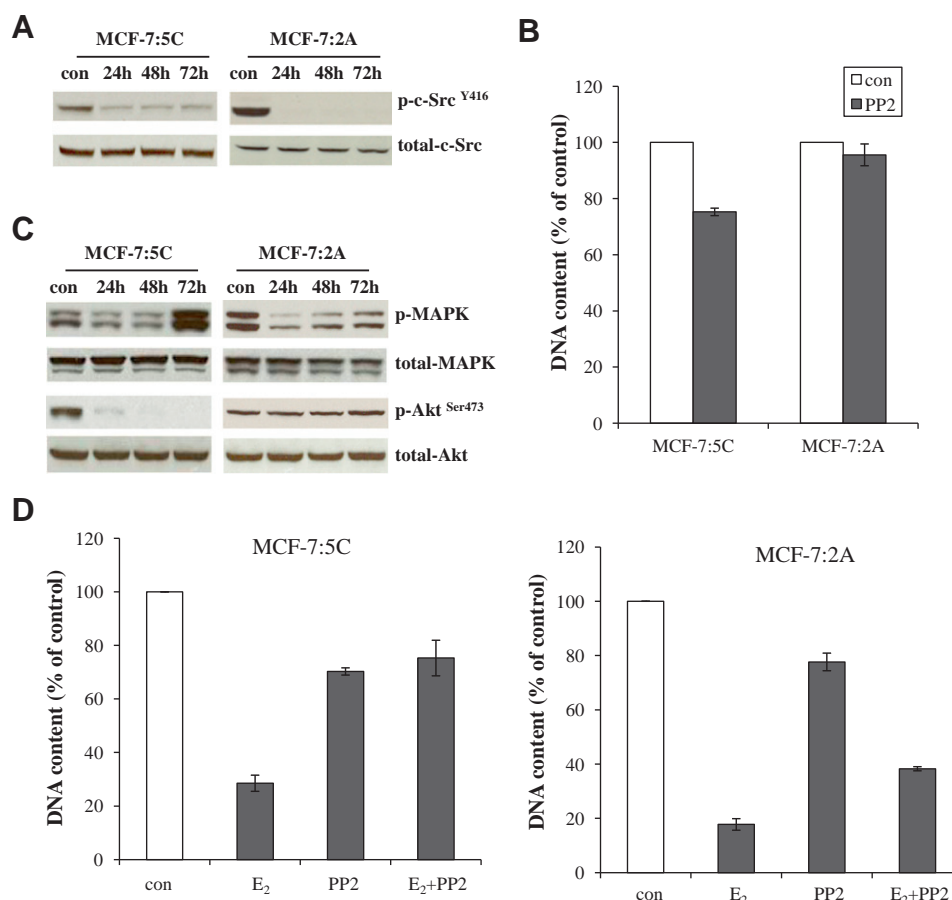


Fig. 3. Effects of the c-Src inhibitor on oestrogen receptor (ER) positive endocrine resistant cell lines. (A) Blocking c-Src phosphorylation in endocrine resistant ER positive cells. MCF-7:5C and MCF-7:2A cells were treated with PP2 (5 μ M) at time points as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (B) Growth inhibitory effects of PP2 on endocrine resistant ER positive cells. MCF-7:5C and MCF-7:2A cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μ M) respectively in culture medium. The cells were harvested after 7 days treatment and total DNA was determined as above. (C) Signalling pathways change in endocrine resistant ER positive cells after PP2 treatment. Cell lysates were harvested as above. Phosphorylated mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt was used for loading controls. (D) The PP2 blocked E₂-induced inhibition in MCF-7:5C and MCF-7:2A cells. MCF-7:5C cells were seeded in 24-well plates as above. After one day, the cells were treated with vehicle (0.1% EtOH), E₂ (10⁻⁹ mol/L), PP2 (5 μ M) and E₂ (10⁻⁹ mol/L) plus PP2 (5 μ M) respectively. The cells were harvested after 7 days treatment and total DNA was determined as above. MCF-7:2A cells were seeded in 6-well plates. After 1 day, the cells were similarly treated as in MCF-7:5C cells. The cells were harvested after 14 days treatment and total DNA was determined as above.

PP2 exerted no inhibitory effects on Sk-Br-3 cells with HER2 overexpression (Fig. 4B). Inhibition of c-Src could efficiently suppress around 60% of cell growth in both resistant cells, MCF-7/F and T47D:C42 (Fig. 4B). The triple negative MDA-MB-231 cell line was the most sensitive to PP2. These results demonstrated that HER2 amplification might be an indicator for resistance to the c-Src inhibitors in clinical trials. Further investigation indicated that PP2 effectively blocked the MAPK and Akt pathways in the c-Src inhibitor sensitive cells, whereas MAPK and Akt phosphorylation were increased in Sk-Br-3 cells (Fig. 4C). The data implied that HER2 might drive the growth pathways in Sk-Br-3 cells.

3.6. Activation status of HER2 determined the inhibitory effects of the c-Src inhibitor

HER2 overexpression leads to a very aggressive cancer phenotype and poor patient survival.²⁷ c-Src is known to bind to HER2 and is thus activated in HER2-overexpressing cancer cells.^{28,29} BT474 and Sk-Br-3 cells overexpress endogenous HER2 (Supplementary Fig. S1B), however, they had different responses to PP2 (Figs. 1B and 4B). To examine whether HER2 activation affects the inhibitory rate of PP2, phosphorylation of HER2 was evaluated. Among tested cell lines, Sk-Br-3, BT474 and T47D:C42 cells had elevated though different levels of HER2 activation. As a

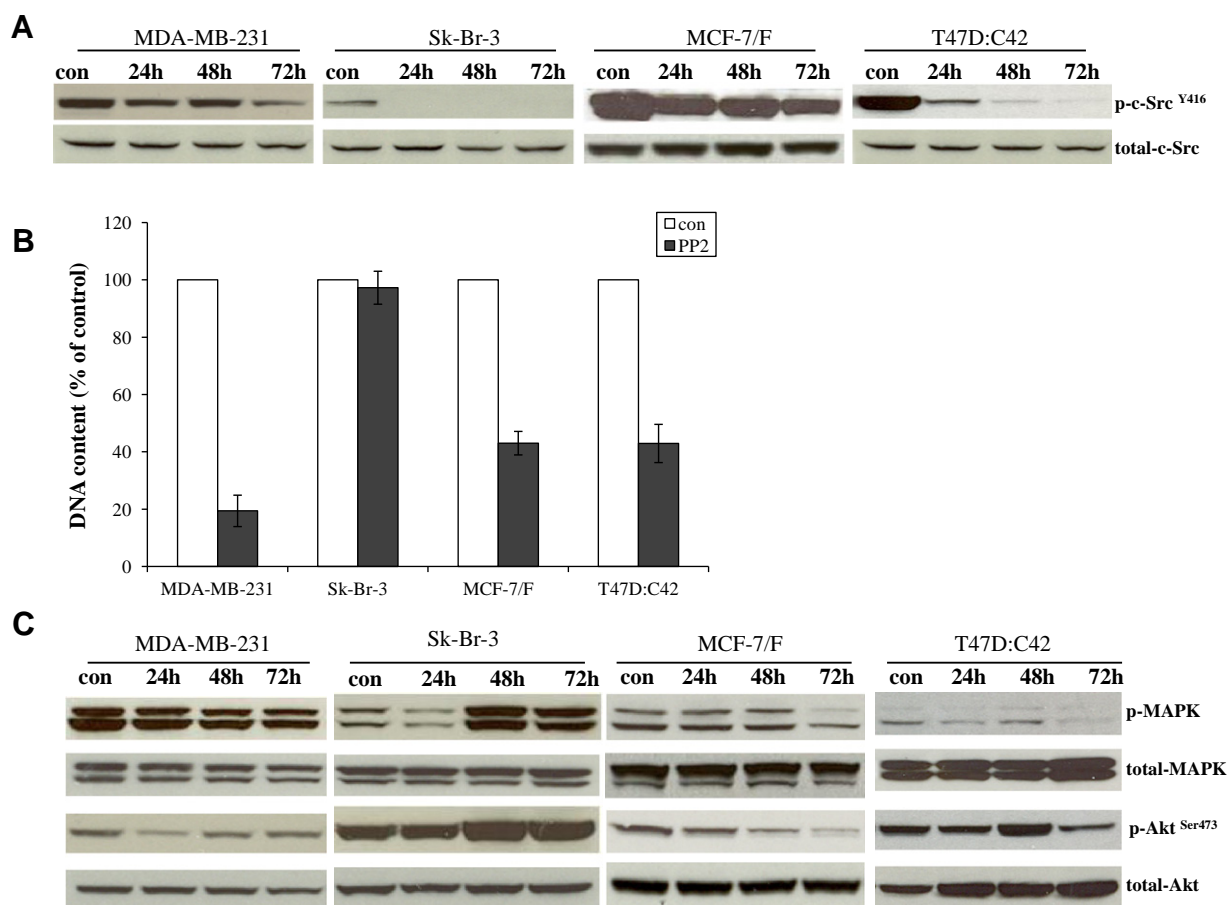


Fig. 4. Effects of the c-Src inhibitor on oestrogen receptor (ER) negative cell lines. (A) Blocking c-Src phosphorylation in ER negative cell lines by PP2. ER negative cells were treated with PP2 (5 μ M) for different times as indicated and cell lysates were harvested. Phosphorylated c-Src was detected by immunoblotting with primary antibody. Immunoblotting for total c-Src was used for loading control. (B) Inhibitory effects of PP2 on ER negative cells. ER negative cells were seeded in 24-well plates in triplicate. After 1 day, the cells were treated with vehicle (0.1% DMSO) and PP2 (5 μ M) in 10% SFS medium. The cells were harvested after 7 days treatment and total DNA was determined as above. (C) Signalling pathways were changed in ER negative cells after PP2 treatment. ER negative cells were treated with PP2 (5 μ M) for different times as indicated and cell lysates were harvested. Phosphorylated mitogen-activated protein kinase (MAPK) and Akt were examined by immunoblotting with primary antibodies. Immunoblotting for total MAPK and Akt was determined for loading controls.

control, HER2 was undetectable in MDA-MB-231 cells (Fig. 5A). HER2 was highly activated in Sk-Br-3 cells compared with BT474 cells which made it hypersensitive to lapatinib, a dual tyrosine kinase inhibitor of HER2 and EGFR (Fig. 5B). The growth inhibitory effects by lapatinib corresponded to the levels of phosphorylated HER2 (Fig. 5B). We observed that HER2 hyper-activation rendered breast cancer cell completely resistant to PP2, the higher HER phosphorylation, the lower responsive rate to PP2 (Fig. 5B). This was further confirmed by S phase changes through flow cytometric analysis (Fig. 5C and Supplementary Fig. S4). Lapatinib reduced S phase in cells with higher HER2 phosphorylation, conversely, PP2 was effective in cells with lower HER2 phosphorylation (Fig. 5C and Supplementary Fig. S4). Lapatinib's antitumour activity was associated with blocking phosphorylation of HER2 and the subsequent inhibition of its downstream signalling pathways (Fig. 5D and Supplementary Fig. S5). Lapatinib

blocked MAPK and Akt pathways in Sk-Br-3 and BT474 cells, but it exerted no inhibition in MDA-MB-231 cells (Supplementary Fig. S5), which demonstrated that antiproliferative effects of lapatinib also correlated with inhibitory ability of growth pathways.

3.7. Blocking c-Src tyrosine kinase recovered ER α expression and reduced HER2 levels in ER negative Sk-Br-3 cells

c-Src may drive oestrogen-dependent ER α proteolysis in a subset of ER negative breast cancer.³⁰ c-Src did not play a critical role in mediating growth pathways in Sk-Br-3 cells (Fig. 4B). To study whether the c-Src inhibitor can regulate ER turn-over in breast cancer cells with HER2 amplification, we found that PP2 could recover ER α expression in Sk-Br-3 cells (Fig. 6A). Real-time PCR analysis showed that mRNA levels of ER α was increased after PP2 treat-

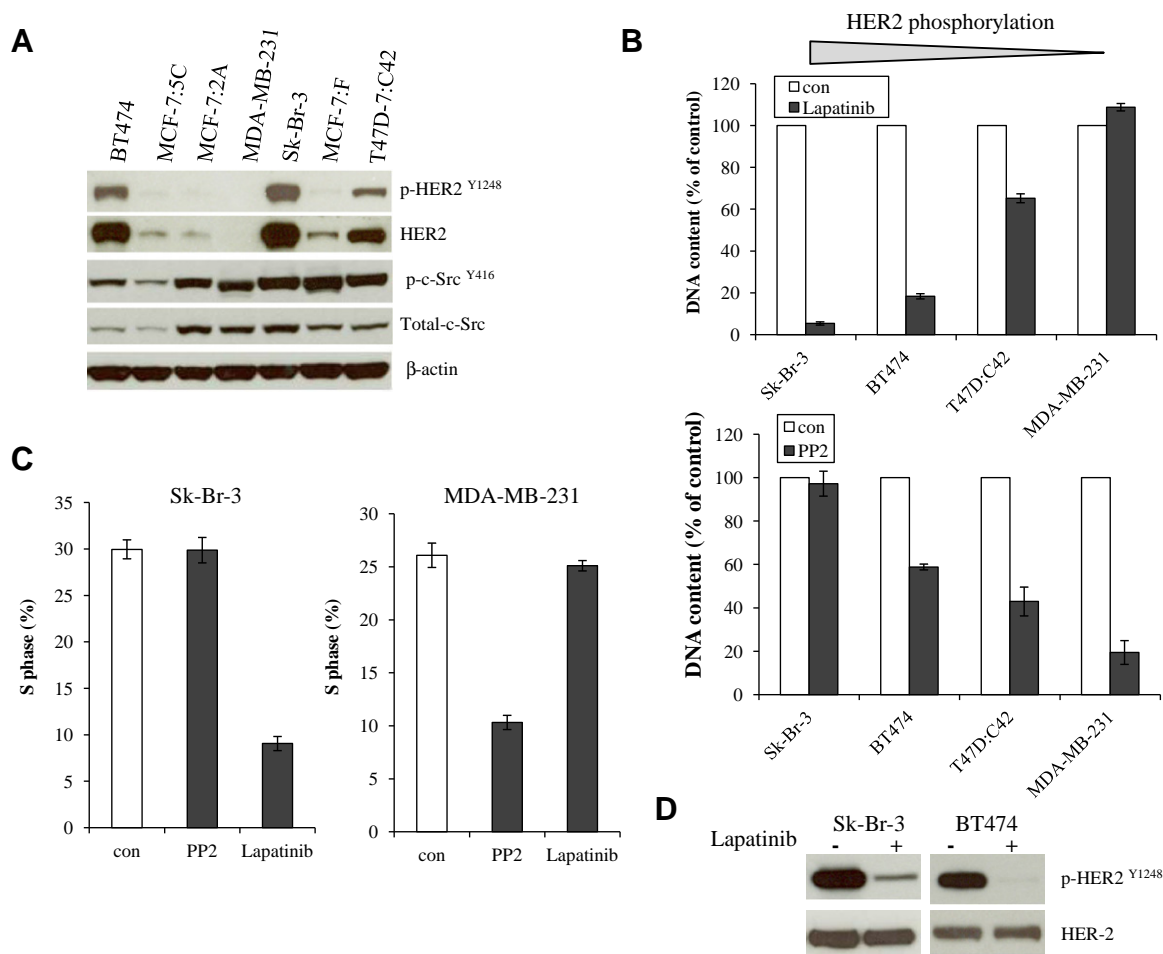


Fig. 5. Activation status of human epidermal growth factor receptor 2 (HER2) determined the inhibitory effects of the c-Src inhibitor. (A) Baseline HER2 phosphorylation in different cell lines. Cell lysates were harvested from different cells. Phosphorylated HER2 and total HER2 were examined by immunoblotting with primary antibodies. Immunoblotting for β -actin was determined for loading control. (B) Inhibitory effects of the HER2 inhibitor and the c-Src inhibitor on cells with elevated HER2 phosphorylation. Sk-Br-3, BT474, T47D:C42 and MDA-MB-231 cells were seeded in 24-well plates in triplicate. After one day, the cells were treated with vehicle (0.1% DMSO), lapatinib (1 μ M) and PP2 (5 μ M) in 10% SFS medium. The cells were harvested after 7 days treatment and total DNA was determined as above. (C) S phase changes after lapatinib and PP2 treatment. Sk-Br-3 and MDA-MB-231 cells were treated with vehicle (0.1% DMSO), lapatinib (1 μ M) and PP2 (5 μ M) for 24 h. Cells were harvested and fixed with 75% EtOH. Cell cycles were analysed through flow cytometry. (D) Blocking HER2 phosphorylation after lapatinib treatment. Sk-Br-3 and BT474 cells were treated with vehicle (0.1% DMSO) and lapatinib (1 μ M) for 24 h. HER2 phosphorylation was examined by immunoblotting with primary antibody. Immunoblotting for total HER2 was determined for loading control.

ment in Sk-Br-3 cells (Fig. 6B) which implied that c-Src was involved in the regulation of ER α not only in the protein level but also at the transcription level. We further demonstrated that PP2 decreased HER2 levels in Sk-Br-3 cells after extending treatment time (Fig. 6C). This result also implied a complicated feedback loop existed between c-Src and HER2 in Sk-Br-3 cells. Importantly, Sk-Br-3 cells acquired responses to 4-hydroxytamoxifen and ICI 182,780 after short-term treatment with PP2 (Fig. 6D and Supplementary Fig. S6). Therefore, it is plausible that the simultaneous interruption of c-Src tyrosine kinase and targeting ER might be an effective treatment for breast cancer cells with HER2 amplification.³¹

4. Discussion

We employed a panel of well characterised breast cancer cell lines (MCF-7, T47D, ZR-75-1, BT474, MDA-MB-231 and Sk-Br-3) and resistant cell lines (MCF-7:5C, MCF-7:2A, MCF-7/F and T47D:C42) to identify biomarkers associated with the inhibitory actions of a specific c-Src inhibitor, PP2. PP2 blocked c-Src tyrosine kinase activity in all cell lines tested. However, the antiproliferative effects of PP2 were associated with the inhibition of ERK/MAPK and/or PI3K/Akt growth pathways. ER positive and HER2 hyperactivation were two important clinically related markers that were associated with the inability of PP2 to inhibit both

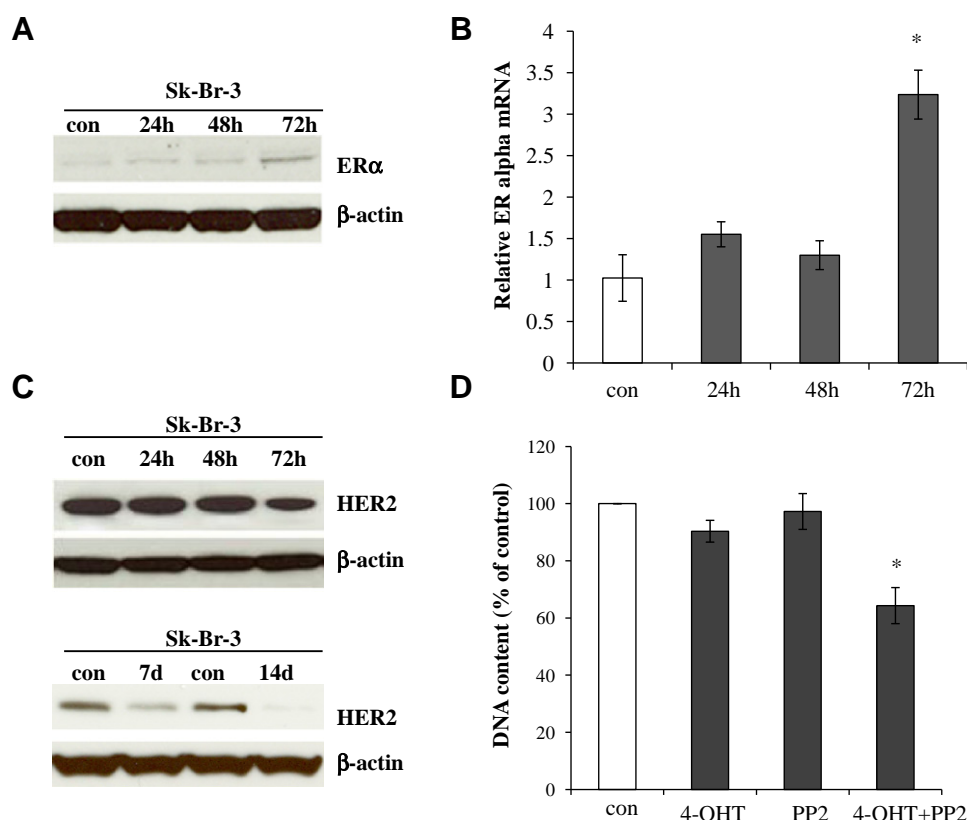


Fig. 6. Blocking c-Src sensitised cell to antioestrogen in Sk-Br-3 cells. (A) ER α expression was elevated in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 as indicated. ER α expression was examined by immunoblotting with primary antibody. Immunoblotting for β -actin was determined for loading control. (B) ER α mRNA was increased in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 (5 μ M) for the times as indicated. RNA was harvested in TRIzol for real-time polymerase chain reaction (PCR) analysis. * P < 0.05, compared with control. (C) HER2 expression was down regulated in Sk-Br-3 cells after PP2 treatment. Sk-Br-3 cells were treated with PP2 for the times as indicated. HER2 was examined by immunoblotting with primary antibody. Immunoblotting for β -actin was determined for loading control. (D) The PP2 sensitised Sk-Br-3 cells to 4-hydroxytamoxifen. Sk-Br-3 cells were treated with vehicle, 4-OHT (1 μ M), PP2 (5 μ M) and 4-OHT (1 μ M) plus PP2 (5 μ M) in 10% foetal bovine serum (FBS) medium. The cells were harvested after 7 days treatment and total DNA was determined as above. * P < 0.05, compared with control.

wild-type and different resistant breast cancer cells. Triple-negative breast cancer cells, defined by a lack of expression of oestrogen, progesterone and HER2 receptors, were the most sensitive to the c-Src inhibitor.

The therapeutic mechanisms of the c-Src inhibitor are to block its phosphorylation and subsequent growth pathways.³² It has been reported that cancer cells which do not manifest detectable c-Src phosphorylation are resistant to the c-Src inhibitor.³³ Generally, cells with higher c-Src activity were more sensitive to PP2 (Fig. 4B), but not all cells with elevated c-Src tyrosine kinase activity were able to be effectively inhibited by the c-Src inhibitor such as ZR-75-1, MCF-7:2A and Sk-Br-3 cells (Figs. 1B and 4B). Thus, the level of c-Src phosphorylation is not sufficient to distinguish responsive cells from cells resistant to the c-Src inhibitor. Growth inhibition also depends on whether c-Src directly mediates growth pathways in a special type of cell. We consistently found that the levels of MAPK phosphorylation and/or Akt phosphorylation were reduced by PP2 in responsive

cell lines but not in resistant cell lines (Figs. 1C, 3C, and 4C).

The non-receptor tyrosine kinase c-Src acts as a critical molecule in relaying ER signalling, including non-genomic and genomic actions.^{4,26} Its activity is modulated by E₂ through multiple mechanisms, leading to breast cancer cell proliferation, invasion and metastasis.^{3,7} Consistently, the growth inhibitory effects by the c-Src inhibitor on ER positive cells appear to be more complex than on ER negative cells in present work. Most ER negative breast cancer cells were sensitive to the inhibition by PP2 (Fig. 4B). However, the majority of ER positive cells were not sensitive to PP2 regardless of whether they were wild-type or long-term oestrogen deprived cells (Figs. 1B and 3B). Although PP2 had moderate ability to inhibit some ER positive wild-type cell growth (Fig. 1B), inhibitory effects by it varied under conditions with or without basal E₂ (Fig. 2C). Our results also demonstrated that c-Src mainly mediated E₂ responses which included E₂-stimulated growth and E₂-

induced apoptosis in ER positive cells (Figs. 2D and 3D). These functions might disturb the therapeutic effects of the c-Src inhibitor on ER positive cells. Although the c-Src inhibitor shows limited activity in ER positive cells as a single-agent, c-Src is consistently activated after ER targeting treatment with tamoxifen^{8–10} which plays a critical role in mediating migration and invasion in tamoxifen resistant cells.^{8,9} Therefore, combined together the c-Src inhibitor and ER blockade may delay endocrine resistance and increase the therapeutic effects.³⁴

The function of c-Src has been linked to its association with the HER2/Neu epidermal growth factor receptor family members.³⁵ In this study, increased expression of EGFR (MDA-MB-231 and MCF-7/F) did not affect the inhibitory effects of PP2, but HER2 overexpression was an indicator for the resistance to PP2 (Fig. 4B). Finn et al.¹⁵ also reported HER2 amplification was a predictive marker for resistance to a c-Src inhibitor, dasatinib, in breast cancer cells. However, both BT474 and Sk-Br-3 cells overexpress endogenous HER2, they had differential responses to PP2 (Figs. 1B and 4B). Further investigation demonstrated that status of HER2 activation determined the inhibitory rate of PP2, the higher HER2 phosphorylation, the lower inhibitory rate of PP2 (Fig. 5B and C). HER2 was highly activated in Sk-Br-3 cells compared with BT474 cells which made it hypersensitive to the HER2 inhibitor but not the c-Src inhibitor (Fig. 5A and B). Therefore, status of HER2 activation may be a better predictive biomarker for resistance to the c-Src inhibitor than currently available total HER2 determined by immunohistochemistry (IHC) or fluorescent *in situ* hybridisation (FISH).¹⁶

The triple negative MDA-MB-231 cells are characterised by a point mutation at codon 13 in the *K-RAS* gene.³⁶ This mutation is responsible for the constitutive phosphorylation of ERK1/2 which leads to a very aggressive cancer phenotype.³⁷ Among tested cell lines, we observed that PP2 could not completely block c-Src phosphorylation in MDA-MB-231 cells within the first 24 h (Figs. 1A, 2B, 3A, and 4A). But the level of c-Src phosphorylation was gradually decreased (Fig. 4A). We prolonged treatment time to 4 days, the level of c-Src phosphorylation was clearly decreased (supplementary Fig. S7). It is unclear how EGFR and *K-RAS* regulate the function of c-Src in MDA-MB-231 cells. The c-Src inhibitor, PP2, effectively suppressed growth in MDA-MB-231 cells, which demonstrated that triple negative breast cancer cells depend on c-Src to proliferate (Fig. 4B). Two independent studies support our observation by showing that the majority of dasatinib sensitive breast cancer cell lines were ‘basal’ type or ‘triple-negative’.^{14,15} The hyper-sensitivity to the c-Src inhibitors provides a good therapeutic option for the clinical triple negative breast cancer (TNBC) patient. However, the TNBC is actually a highly diverse group of cancer,³⁸ so

that the determination of ER, PR and HER2 is not a precise classification to subtype this aggressive disease. MDA-MB-231 cells can not represent clinical TNBC model. Recent Phase II clinical trial shows that single-agent dasatinib has limited activity in unselected patients with TNBC,¹⁷ which suggests that a strategy of better patient selection with gene signatures is required to further evaluate the potential of the c-Src inhibitors in TNBC patient.³⁸

In summary, this study demonstrated a complex association exists among ER, HER2 and c-Src in different breast cancer cell lines. Moreover, our results underscored that ER expression and HER2 overexpression (especially over-activation) might be causes of resistance to a c-Src inhibitor in breast cancer. Our findings may be of value for future clinical investigation to determine the therapeutic efficacy of c-Src inhibitors in ER negative breast cancer with or without HER2 over-activation.

Role of the funding source

Ping Fan, Russell E. McDaniel and Helen R. Kim’s salaries, as well as laboratory supplies supported by the following: the Department of Defense Breast Program under award number W81XWH-06-1-0590 Center of Excellence (VCJ); subcontract under the SU2C (AACR) Grant No. SU2C-AACR-DT0409 (VCJ); the Susan G. Komen For The Cure Foundation under Award number SAC 100009 (VCJ) and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008 (VCJ).

Conflict of interest statement

None declared.

Acknowledgements

This work (VCJ) is supported by the Department of Defense Breast Program under Award number W81XWH-06-1-0590 Center of Excellence; subcontract under the SU2C (AACR) Grant number SU2C-AACR-DT0409; the Susan G Komen For The Cure Foundation under Award number SAC100009; GHUCCTS CTSA (Grant # UL1RR031975) and the Lombardi Comprehensive Cancer Center Support Grant (CCSG) Core Grant NIH P30 CA051008. The views and opinions of the author(s) do not reflect those of the United States Army or the Department of Defense.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejca.2012.04.020>.

References

1. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* 2007;**72**:7–25.
2. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;**344**:783–92.
3. Thomas SN, Brugge JS. Cellular functions regulated by Src family kinases. *Ann Rev Cell Dev Biol* 1997;**13**:513–609.
4. Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci USA* 2002;**99**:14783–8.
5. Belsches-Jablonski AP, Biscardi JS, Peavy DR, et al. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 2001;**20**:1465–75.
6. Bromann PA, Korkaya H, Courtneidge SA. The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* 2004;**23**:7957–68.
7. Shupnik MA. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* 2004;**23**:7979–89.
8. Hiscox S, Morgan L, Green TP, et al. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2006;**97**:263–74.
9. Zhao Y, Planas-Silva MD. Mislocalization of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity. *Cancer Lett* 2009;**275**:204–12.
10. Fan P, Wang J, Santen RJ, Yue W. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. *Cancer Res* 2007;**67**:1352–60.
11. Zhang S, Huang WC, Li P, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nat Med* 2011;**17**:461–9.
12. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 2006;**354**:2531–41.
13. Demetri GD, Lo Russo P, Macpherson IR, et al. Phase I dose-escalation and pharmacokinetic study of dasatinib (BMS-354825), a Src and multi-kinase inhibitor, in patients with advanced solid tumors. *Clin Cancer Res* 2009;**15**:6232–40.
14. Huang F, Reeves K, Han X, et al. Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. *Cancer Res* 2007;**67**:2226–38.
15. Finn RS, Dering J, Ginther C, et al. Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/"triple-negative" breast cancer cell lines growing in vitro. *Breast Cancer Res Treat* 2007;**105**:319–26.
16. Finn RS, Bengala C, Ibrahim N, et al. Dasatinib as a single agent in triple-negative breast cancer: results of an open-label phase 2 study. *Clin Cancer Res* 2011;**17**:6905–13.
17. Mayer E, Baurain JF, Sparano JA, et al. A phase 2 trial of dasatinib in patients with advanced HER2-positive and/or hormone receptor-positive breast cancer. *Clin Cancer Res* 2011;**17**:6897–904.
18. Chen Y, Guggisberg N, Jorda M, et al. Combined Src and aromatase inhibition impairs human breast cancer growth in vivo and bypass pathways are activated in AZD0530-resistant tumors. *Clin Cancer Res* 2009;**15**:3396–405.
19. Pink JJ, Jordan VC. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* 1996;**56**:2321–30.
20. Jiang SY, Jordan VC. Growth regulation of estrogen receptor negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J Natl Cancer Inst* 1992;**84**:580–91.
21. Jiang SY, Wolf DM, Yingling JM, Chang C, Jordan VC. An estrogen receptor positive MCF-7 clone that is resistant to antiestrogens and estradiol. *Mol Cell Endocrinol* 1992;**90**:77–86.
22. Pink JJ, Jiang SY, Fritsch M, Jordan VC. An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995;**55**:2583–90.
23. Murphy CS, Pink JJ, Jordan VC. Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Res* 1990;**50**:7285–92.
24. Liu H, Cheng D, Weichel AK, et al. Cooperative effect of gefitinib and fumitremorgin c on cell growth and chemosensitivity in estrogen receptor alpha negative fulvestrant-resistant MCF-7 cells. *Int J Oncol* 2006;**29**:1237–46.
25. Lewis JS, Meeke K, Osipo C, et al. Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Natl Cancer Inst* 2005;**97**:1746–59.
26. Faivre EJ, Lange CA. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. *Mol Cell Biol* 2007;**27**:466–80.
27. Yu D, Hung MC. Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. *Oncogene* 2000;**19**:6115–21.
28. Muthuswamy SK, Muller WJ. Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene* 1995;**11**:1801–10.
29. Belsches-Jablonski AP, Biscardi JS, Peavy DR, et al. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 2001;**20**:1465–75.
30. Chu I, Arnaout A, Loiseau S, et al. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. *J Clin Invest* 2007;**117**:2205–15.
31. Page K, Hava N, Ward B, et al. Detection of HER2 amplification in circulating free DNA in patients with breast cancer. *Br J Cancer* 2011;**104**:1342–8.
32. Luttrell DK, Lee A, Lansing TJ, et al. Involvement of pp 60c-src with two major signaling pathways in human breast cancer. *Proc Natl Acad Sci USA* 1994;**91**:83–7.
33. Okamoto W, Okamoto I, Yoshida T, et al. Identification of c-Src as a potential therapeutic target for gastric cancer and of MET activation as a cause of resistance to c-Src inhibition. *Mol Cancer Ther* 2010;**9**:1188–97.
34. Chen Y, Alvarez EA, Azzam D, et al. Combined Src and ER blockade impairs human breast cancer proliferation in vitro and in vivo. *Breast Cancer Res Treat* 2011;**128**:69–78.
35. Tan M, Li P, Klos KS, et al. ErbB2 promotes Src synthesis and stability: novel mechanisms of Src activation that confer breast cancer metastasis. *Cancer Res* 2005;**65**:1858–67.
36. Eckert LB, Repasky GA, Ülkü AS, et al. Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res* 2004;**64**:4585–92.
37. Toulany M, Baumann M, Rodemann HP. Stimulated PI3K-AKT signaling mediated through ligand or radiation-induced EGFR depends indirectly, but not directly, on constitutive K-Ras activity. *Mol Cancer Res* 2007;**5**:863–72.
38. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011;**121**:2750–67.

siRNA SCREENING: A PROCESS MODEL TO EVALUATE HIT RATE DISCOVERY

Yoganand Balagurunathan¹, Spyro Mousset² and ¹Michael L. Bittner¹

¹Integrated Cancer Genomics/Computational Biology, and ²Cancer Drug Development Laboratory

The Translational Genomics Research Institute, Phoenix, AZ 85004.

Email: yoga@tgen.org

ABSTRACT

RNA interference has been widely used to identify genes involved in the production of particular biological phenotypes. This type of gene silencing technology has been used in plants, invertebrates and mammalian systems [1]. The availability of the sequences of large numbers of genes has allowed large libraries of siRNAs to be produced. To effectively use these libraries in screens, high-throughput robotic screening methodologies have been devised. The identification of meaningful results from any screening system requires the analyst to identify and discount variances in output that arise from the devices used in the screen as well as variances that arise from biological components in the screen that are unrelated to gene silencing. In this developing technology, this analytical task is made difficult by variances in uptake of the siRNAs by the cells, variations in the magnitude of the silencing effect, and mechanical effects that can produce systematic alterations in cell delivery and cell growth during the experiment. To examine how the analysis can be optimized, models of the screening process have been built using estimates of the various noise and signal variances derived from available screen data. Synthetic data was then generated from this model and used to test the capability of a number of data normalization methods to reduce noise and allow signal detection.

1. INTRODUCTION

RNA interference is a process by which the functionality of a gene is silenced by introducing short RNA oligonucleotides whose sequence is complementary to a specific region of the mRNA of the target gene. In theory any gene can be silenced, so these methods provide a broadly applicable way to study the phenotypic consequences of the loss of a particular gene's function. As this assay can be readily carried out in parallel if the assay readout is a simple, optically accessible endpoint, it has been exploited in screens where, single or multiple target-specific siRNA species are applied to cells in individual wells of a microtiter plate. The ability to design the silencing RNA is not perfect, any individual species has an approximately 30% chance of reducing the mRNA levels by 70%, so it is common practice to use several different species for each gene to be screened. In order to screen tens of thousands of genes, high-throughput methods that borrow heavily from methodologies used for drug screening have been implemented. Drug screening has been widely used, and a variety of

methods have been proposed for noise reduction in these types of screen [2].

It is typical in engineering to build models to study process noise. These models are then used to study the downstream effects of both the noise and proposed noise reduction methods on analytic accuracy. Modeling studies have been applied to optimize the analysis of high-content array technology [3]. Understanding the variances in processes carried out in biological systems is not an easy task, since there are usually several types of noise-producing sources to be dealt with. It has been helpful in some cases to make approximate data models that allow mathematical descriptions of the different sources to be individually specified, and then operate the aggregate model using different settings for the various parts to more clearly see how various noise and normalization treatments impact analytical accuracy. This type of approach allowed the development of improved data extraction methods for gene expression from microarrays [4]. Models for siRNA high-content screening technology would give greater insight both for understanding the behavior of the cells in the assay and of the characteristics of the screening procedure.

The proposed model of siRNA screening attempts to generate synthetic data where all of the known sources of signal and noise are represented. The model is stochastic with appropriate statistical distributions assigned to its controlling parameters. This type of approach is aimed at identifying the best data extraction procedures. This will reduce Type I (false positive) errors and Type II (false negative) errors. In this work five different standardization methods are tested using synthetic data for which the ground truth is known, making it possible to effectively evaluate true and false hit rates against presence of variable amounts and types of noise.

2. PROCESS MODEL

This process is modeled against a high throughput robotic system that deposits cells, siRNA species and drugs into the wells of a series of 384 well plates. After a prescribed period of incubation at a controlled temperature a luminescent reporter whose output is proportional to the number of live cells is added and then a photometer quantifies the luminescence. The typical assay screens for genes whose functional inactivation allows exposure to a level of drug that would normally produce only a small reduction in cell growth to achieve a very high reduction in cell growth, identifying genes that potentiate drug response. In the current model we assume

that unperturbed cells grow evenly in all the wells, that introduction of an effective small siRNA sequence will achieve 80% or higher silencing and that most of the genes silenced are not a part of the process that the drug targets and therefore do not exhibit any reduced (or enhanced) cell growth phenotype. The model also assumes that a modest number of gene silencing events result in a significant change in cell growth. At each of the assay stages, distributional randomness will account for variabilities that may occur in reality. Our focus will be on modeling the resultant luminescent level indicating cell growth/survival that arise from the differing treatments applied in this assay. A complete assay reports luminescence for untreated cells, drug treated cells, siRNA treated cells and drug plus siRNA treated cells.

The system is modeled statistically with appropriate distribution to simulate the effects each of these four conditions will produce on cell growth. We will describe the modeling of these changes in this section. At the start, before any treatment, the cells in the treated well will be very similar to untreated, control cells. A normal distribution with set mean would be an ideal approximation, where U and T are the untreated and treated intensities. At the beginning of an experiment, raw cells are expected to follow a normal distribution.

$$U \sim N(\mu_g, \sigma_g) \quad (1)$$

Process 1: No siRNA, No Drug, in this process the treated and untreated observation is expected to be the same as no treatment is introduced, but the system of observation will have a noise modeled by a additive gaussian.

$$U_1 = U + N(\mu_n, \sigma_n) \quad (2)$$

$$T_1 = U + N(\mu_n, \sigma_n) \quad (3)$$

$$\text{Where } \mu_n = 0, \sigma_n = k_n E[U]$$

Process 2: Addition of siRNA; is expected to affect selective genes and in most cases the siRNA will not affect growth/survival, meaning there will be no observable effect. In those cases where siRNA silences a gene that alters the cell's intrinsic growth or survival, it is expected to change the number of cells present when the assay is read out. The most common result, when there is an effect, is a reduction in growth/survival, leading to decreased luminescence. These changes due to siRNA can be modeled by discrete events selected by probability of chance, which are: positive siRNA silencing (decreased luminescence, above $p_1=15\%$ chance), no change (over $p_2=80\%$ chance), and increased luminescence (in a small number of cases, $p_3=5\%$ chance). The probabilities indicated are the levels observed in most cases. If an siRNA event is chosen to happen then the amount of change is defined by a factor ρ_R , a random variable made to follow an exponential distribution. This produces the extent of change (enhancement or decrement) in the small number of cases where silencing has a large effect. The magnitude of change will be a fold change, factored by a power factor m_R , which can take values from: [$r_1=-0.15$,

$r_2=0$, $r_3=0.05$] selected by discrete probabilities. A positive scale factor will mimic silencing effects decreasing growth/survival and a negative scale factor will model enhanced growth/survival. In addition any experimental repetition of the same treatment would result in some variation modeled by an additive noise. The Untreated (no addition) and Treated observations with the addition of siRNA will then be:

$$U_2 = U + N(\mu_n, \sigma_n) \quad (4)$$

$$T_2 = (\rho_R)^{m_R} T_1 + N(\mu_n, \sigma_n) \quad (5)$$

Where $\rho_R \sim \text{Exp}(\Xi_R)$, $m_R \in \{r_1, r_2, r_3\}$ with $P_R \in \{p_1, p_2, p_3\}$

Process 3: Addition of drug to the cells will have a small and negative effect on cell growth with some amount of randomness to emulate experimental conditions. This change is modeled by a small decrement in growth believed to uniformly affect all the cells which is modeled by a shift k_D ($k_D < 1$). This process is made to follow a normal distribution to mimic a small change within the plate, the scale factor k is made a small percentage to the mean. The drug effect will then be.

$$U_3 = U + N(\mu_n, \sigma_n) \quad (6)$$

$$T_3 = (1 - k_D) T_1 + N(\mu_n, \sigma_n) ; \quad (7)$$

$$\text{Where } k_D \sim N(\mu_{K_D}, \sigma_{K_D}), \sigma_{K_D} = k_{s_d} \mu_{K_D}$$

Process 4: Addition of siRNA and Drug to the experiment will in most cases result in cumulation of individual effects. This is modeled as a multiplicative effect that happens in most cases (occurrence P_{rc} set to 80%). A silenced gene that enhances drug efficacy is expected to be highly detrimental to the cell's mortality, producing a considerable reduction in luminescent level. This synergistic effect is modeled as a multiplicative factor ($1/k_c$), where the scale k_c has a several events with discrete probability of occurrence.

$$U_4 = U + N(\mu_n, \sigma_n) \quad (8)$$

$$T_4 = f_R(t) T_1 + N(\mu_n, \sigma_n) ; t \sim U(0,1) \quad (9)$$

$$\text{where } f_R(t) = \begin{cases} (1 - k_D)(\rho_R)^{m_R} ; t \leq P_{rc} \\ (1 - k_D)(\rho_R)^{m_R} (1/k_c) ; o.w \end{cases}$$

$$k_c \in \{s_1, s_2, s_3, s_4, s_5, s_6, s_7\}$$

$$\text{with } P_c \in \{ps_1, ps_2, ps_3, ps_4, ps_5, ps_6, ps_7\}$$

The proposed model is thus quite flexible. Each source of signal and noise for each type of treatment is individually modeled as random; with an appropriate probability distribution and the overall model can be adapted for any further type of change as the high throughput system evolves. Typical data output is shown in Figure 1. Where the model is simulating the process at set levels of additive experimental noise factor (k_n).

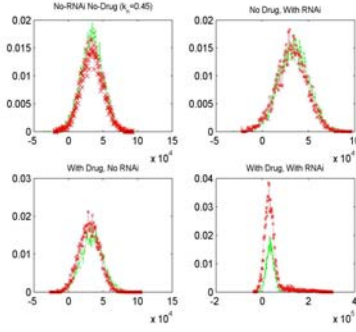


Figure 1. Distribution of the process (overall) with no normalization, noise levels at $k_n = 0.45$.

3. STANDARDIZATION METHODS

In the recent years the evolution of high-throughput technologies have led to broad use of normalization methods in biological assays such as expression arrays, SNP arrays, protein mass spectrometry, etc. Each of these multi-analyte assays have different experimental and biological sources of noise to contend with. siRNA screens have some similar and some different issues than other biological assays. The highest variations in siRNA screening results have tended to derive mainly from two sources. The delivery of cells to the assay plates is somewhat complicated by the tendency of the cells to settle in the media, which can lead to systematic variations when many plates are processed. Another technically troublesome problem is variance in the rate of uptake of the siRNA during transfection, which can be systematic or sporadic, and varies on a cell line by cell line basis. The first variation is relatively easier to correct based on observing the variations within and among the plates. The variance in level of silencing is harder to account for as these type of changes are less systematic. In our analysis we examine four types of normalization methods to correct for the systematic errors, namely: (a) plate-wise median, (b) median polish (c) B-score (d) regression based correction (1st & 2nd order).

3.1 Plate-wise median: A median value at plate level was computed and used as a standardization factor to rank all the luminescence levels individually for untreated and treated observations. This method compensates for the plate level changes but is known to shrink the range of the data while being immune to outliers. It's a popular approach used in many biological plate-based screens to compensate for systematic errors. The observation for a plate p will be computed, where x_i would be 'untreated' or 'treated' observation for i^{th} position for p^{th} plate or screen, $x'_i = (x_i / \text{median}(X_p))$

3.2 Median polish: This is a variation of 2-way ANOVA first proposed by John Tukey [5] to be immune to outliers, by using medians instead of means. In a data set where the row effect is independent of column effect with the assumption that the change is additive in nature, median polish method corrects the variations by obtaining the residues along either direction. It is a classical method known to be resistant to outliers. The row and column estimates are obtained by iteratively subtracting the group effects by taking the median along each direc-

tion. In our experiment, the median polish residues obtained individually for untreated and treated observation for each plate, we limited to one iteration along either direction. If U and T are untreated and treated observation for a plate p , the two-way compensated observation will be

$$U'_p = (U_p - \hat{U}_p) \quad (10)$$

$$T'_p = (T_p - \hat{T}_p) \quad (11)$$

Where \hat{U}_p and \hat{T}_p residues were obtained using the additive data model. This type of standardization as a method is adapted to siRNA screening in this work.

3.3 BScore: The B-score (*Better score*) was first proposed by Brideau et.al [2] to compensate systematic errors in high throughput drug screens. It was assumed that the drug screens had independent row/column effects with the assumption that the noises are additive. The plate-wise screens were subject to window smoothing along row and column to even out variations and followed by a median polish to obtain residues. The procedure is repeated until the residues converge; in most cases they converge in few runs. In order to save computational time with out compromising the polish, an upper limit was set on the number of iterations ($I_t = 5$). These residues were then divided by the median absolute deviation for the screen to standardize per screen or plate. This method compensates for outliers effectively but the smoothing process seems to hide scattered individual changes. If $e'_{ij,p}$ is the residue obtained after smoothing

for a plate p , then *Bscore* is defined as $e'_{ij,p} / MAD_p$, where MAD_p is the median absolute deviation of the residues for a plate p .

3.4 Regression: The regression methods are widely used in microarray analysis to correct the channel variations, between experiment and control [6]. We use this approach in the siRNA model study with the basic assumption that the change in untreated and treated samples tend to have same distributional trend and the scatter plot will make them stay on the 45 degree line. Any deviation from the scatter is due to systematic variations and such variations corrected by minimizing the mean square error between them. If U and T be the untreated and treated samples for a plate p then the coefficients are obtained by minimizing,

$$E[(U_p - T_p)^2] = E[(U_p - (a_2 T_p^2 + a_1 T_p + a_0))^2] \quad (12)$$

In our study both first order (a_1, a_0) and second order (a_2, a_1, a_0) coefficients are computed. All the regression correction is carried out plate-wise. The method is adapted to siRNA screening using experience from expression arrays [6].

4. HIT DISCOVERY AND RESULTS

The process model is simulated with some global parameters estimated from the real data (plate mean, plate

spread) and the performance of the normalizations are evaluated to determine their effects on hit rates against various noise types and levels. Most parameters could not be estimated due to unavailability of control experiments. To offset, the controlling parameters are made to follow a distribution which makes the study relevant for a possible range of true variation. Once the model process is generated for each of the conditions (-siRNA/-Drug; +siRNA/-Drug, -siRNA/+Drug, siRNA/+Drug) the difference is computed between the untreated and treated conditions for each condition. A confidence level (at 99% or $\sim 3\sigma$) was obtained for the difference distribution of untreated to treated under the No-siRNA, No-Drug condition. This limit was used to mark a gene as a change or a hit (Treated>Untreated or treated<Untreated). It is widely believed that the ability to accurately determine a hit will decay as the level of model noise increases. Figure 2 shows the entire system model and Table 1. lists the parameters used for the model simulation.



Figure 2. siRNA process model

For these evaluations the process is repeated large number of times ($N_R=1000$) and the ratio of hits found by analysis to the ground truth number of generated hits is found for each repeat and reported. This ratio gives a measure of system accuracy. The hit-ratio is averaged over all the repeats. A glimpse of the main inferences is provided in Figure 3, showing the average hit rate for With-siRNA, With-Drug at two different experimental noise level (α_n), plotted for various levels of enhancement. In this case the confidence level set by the No-siRNA, No-Drug condition, but the model performance could be evaluated with different types of treatments. It is interesting to observe that the hit discovery rate decreases with the increase in additive noise or equivalently experimental variability, in some cases noise seem to help in the discovery. At low noise levels (first panel) experiment behaves as expected, the average hit rate spreads evenly in either direction of the enhancement (in log scale, $\log 1=0$). It is interesting to note poor performance of B-Score and median polish mainly attributable to the data fitting procedure for B-score, which may not be appropriate for siRNA screens. Median polish may remove system variations more than needed. Regression and median scaling seem to perform equally well. As the noise level increases; at lower negative enhancement levels hit-rate seem to be poor. Additive noise seems to aid positive enhancement factors, it is to be noted that negative enhancement (or reduction in growth) is of interest in siRNA studies. As expected, at high levels of enhancement most methods perform well, even without standardization. It is to be noted, at the

highest levels of enhancement of the effect of the silencing event, the hit rate plots drop to zero. This is not due to non-recognition of the events, but to their improbability in the model. The probability of these very large changes is quite low and there are frequently no point modeled in these ranges. We are in the process of estimating the parameters from the real experiments and hope to carryout model studies to better understand the system.

ACKNOWLEDGEMENTS

The Authors would like to thank Dr. Edward. R. Dougherty for his valuable comments on the analysis and the members of the TGen Cancer Drug Development Laboratory, especially Dr. Qiang Que and Dr. David Azorsa for actively sharing their experience in collecting and evaluating screening data.

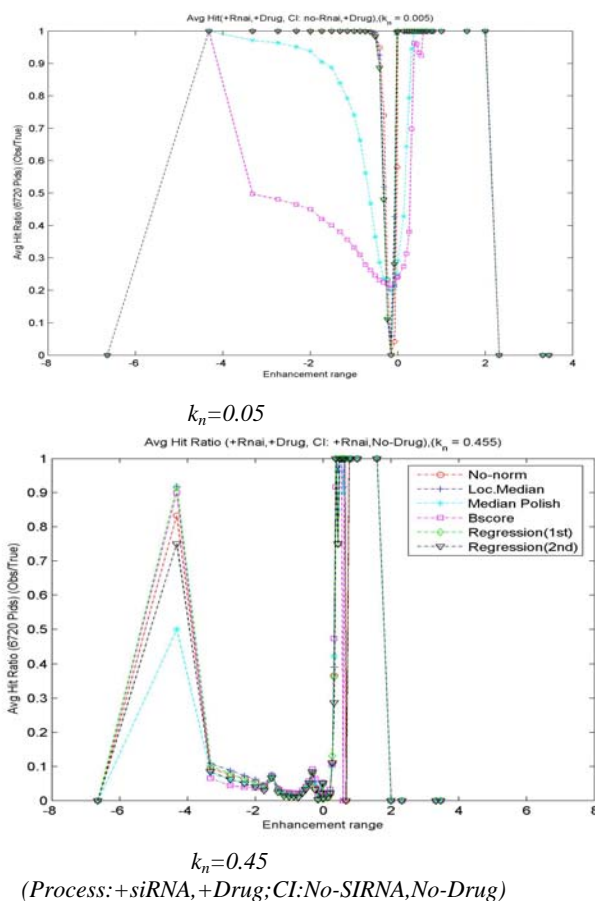


Figure 3. Average hit ratio of observed to true markers using confidence level set by No-siRNA, No-Drug experiment for +siRNA,+Drug condition at two different noise levels. The green line (with +) shows the hits discovered with no-standardization, hits with local median standardization is shown in blue (with +), hits with median-polish is shown in cyan (with *), hits with B-score standardization is shown in magenta (with square), hits with regression (1st order) is shown in yellow (with diamond), hits with regression (2nd order) is shown in black (with down triangle).

Table 1. Parameter setting for the siRNA process simulation
<p>(i) $N_{sample} = 6724$</p> <p>(ii) <i>Sample Level</i>: $\mu_g \sim U[31782, 35313]$; $\sigma_g = K_g \mu_g$; $K_g \sim U[0.05, 0.20]$</p> <p>(iii) <i>Additive Noise</i>: $\mu_n = 0$; $k_n = \{0.05, \Delta n = 0.05, 0.45\}$</p> <p>(iv) <i>siRNA effect</i>: $\Xi_R \sim U[a_R, b_R]$; $[a_R = 50, b_R = 100]$ $m_R \in \{-0.15, 0, 0.05\}$ with $P_R \in \{0.15, 0.8, 0.05\}$</p> <p>(v) <i>Drug effect</i>: $\mu_{k_d} \sim U[0.1, 0.15]$; $k_{s_d} \sim U[0.05, 0.1]$</p> <p>(vi) <i>Drug with siRNA effect</i>: $P_{rc} = 0.8$; $k_c \in \{s_1, s_2, s_3, s_4, s_5, s_6, s_7\} = \{1, 1.5, 2, 3, 4, 5, 7\}$ $P_c \in \{ps_1, ps_2, ps_3, ps_4, ps_5, ps_6, ps_7\}$ $= \{0.4, 0.1, 0.1, 0.1, 0.1, 0.1, 0.1\}$</p> <p>(vii) <i>Repeats</i>: $N_R = 1000$; (viii) <i>Control Probes</i>: $N_{cp} = 0.1$</p>

5. REFERENCES

1. Mittal. V., Improving the efficiency of RNA interference in mammals, Nature genetics (reviews), vol.5, 2004.
2. Brideau. C., Gunter. B., Pikounis. B., Liaw. A., Improving statistical methods for hit selection in high-throughput screening, Journal of Biomolecular screening, 8(6), 2003.
3. Balagurunathan, Y., Dougherty, E. R., Chen, Y., Bittner, M. L., and J. M. Trent, Simulation of cDNA Microarrays via a Parameterized Random Signal Model, *Biomedical Optics*, Vol. 7, No. 3, 507-523, 2002.
4. Y. Chen, E. R. Dougherty, and M. L. Bittner, Ratio-Based Decisions and the Quantitative Analysis of cDNA Microarray Images, *Journal Of Biomedical Optics* 2(4), 364–374, 1997.
5. J. Tukey. *Exploratory Data Analysis*. Addison-Wesley, Reading (MA), 1977.
6. Hua. J., Balagurunathan. Y., Chen. Y., Lowey. J., Bittner. L. M., Xiong. Z., Suh. E., Dougherty. E. R., Normalization benefits microarray-based classification, *Eurosis Journal of bioinformatics and system biology*, vol.1, 1-13, 2006.